MOLECULAR TECHNIQUES FOR THE DETECTION OF COLORECTAL CANCER CELLS IN THE PERITONEAL CAVITY

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All the work described in this thesis has been performed by me, in the laboratory and under the supervision of Dr Alan Morris.

DECLARATION

None of the material contained in this thesis has previously been published, or been submitted for another degree.

SUMMARY

Colorectal cancer is a major health problem, and many patients relapse despite apparently curative treatment. Local recurrence is an important factor, and is more common when cancer cells are present on the free serosal surface or circumferential resection margin. We hypothesised that detecting tumour cells in the peritoneal cavity during surgery (in peritoneal washings) or post-operatively (in drain fluids) would act as a marker for risk of local recurrence, and that the use of molecular biological techniques would allow for the sensitive detection of such cells.

We collected samples of colorectal tumours, with washings from the peritoneal cavity at the start and end of surgery, and fluid from the surgical drain on the first and second post-operative day. Epithelial cells in the peritoneal samples were enriched using magnetic cell separation (MACS). Using mutation specific PCR or a Mismatch Ligation Assay we detected common mutations of *K-ras, TP53, APC* and *BRAF* in primary tumour samples, and when such a mutation was found, we studied the peritoneal samples from that patient for the same mutation.

We detected 23 mutations in 22 (out of 46) tumours from 21 patients. In 16 patients, at least one of the peritoneal samples gave a positive result for the same mutant DNA. Using MACS increased the proportion of positive samples. Half of the patients with positive peritoneal samples had Dukes' stage A or B tumours. Follow up is not yet long enough to allow conclusions to be drawn on the significance of these results.

We have described techniques allowing mutations to be characterised in half of colorectal tumours and have demonstrated the presence of cells with the same mutation in peritoneal samples from three-quarters of patients. Longer follow up will show whether our tests are too sensitive, or whether they provide useful information about likely local recurrence.

LIST OF ABBREVIATIONS

Italicised terms are gene names

APC	adenomatous polyposis coli gene, frequently mutated in CRC (*q.v.*)
APR	abdomino-perineal excision of rectum
ATCC	American type culture collection
A&W	alive and well
Bat-26	marker of microsatellite instability
Ber-EP4	epithelial-specific membrane glycoprotein
bp	base pairs
BRAF	one of the *RAF* (*q.v.*) genes encoding kinase regulated by ras (q.v.)
°C	degrees Celsius
cDNA	complementary DNA
CEA	carcino-embryonic antigen
CK	cytokeratin
CRC	colorectal cancer
CT	computed tomography
CTNNB1	gene coding for β-catenin
dNTPs	mixture of deoxyribonucleotides
EDTA	ethylene diamine tetra-acetate
FAP	familial adenomatous polyposis, inherited condition involving *APC* (*q.v.*)
FCS	foetal calf serum
GDP	guanosine diphosphate
GTP	guanosine trisphosphate
HEA	human epithelial antigen
hMLH1	human MutL homologue 1
hMSH2	human MutS homologue 2
HNPCC	hereditary non-polyposis colorectal cancer, not involving *APC* (*q.v.*)
HTCF4	human T cell factor 4

IgG	immunoglobulin G
inHg	inches of mercury
kb	kilobases
K-ras	Kirsten ras, one of the *ras* (*q.v.*) family of oncogenes
L-15	tissue culture medium
LPI	local peritoneal involvement
M	metastases (in TNM (*q.v.*) staging)
MACS	magnetic activated cell separation
MAP	mitogen activated protein
MASA	mutant allele specific amplification
MDM2	a regulator of p53 (*q.v.*)
µg	microgram
mg	milligram
MI	myocardial infarction
µl	microlitre
ml	millilitre
MLA	mismatch ligation assay
mM	millimolar
mmHg	millimetres of mercury
MMR	mismatch repair
MSI	microsatellite instability
MSS	microsatellite stable
N	nodes (in TNM (*q.v.*) staging)
NEG	negative
ng	nanograms
oligo	oligonucleotide
^{32}PγATP	adenosine triphosphate with ^{32}P-phosphate at the γ position
p21^{ras}	product of *ras* (*q.v.*) oncogene
p53	tumour suppressor protein, product of *TP53* (*q.v.*) gene
p105RB	retinoblastoma protein
PBS	phosphate-buffered saline

PCR	polymerase chain reaction
PET	positron emission tomography
POD	pouch of Douglas
polydA	polydeoxyadenylic acid
POS	positive
POST-OP	post-operative (washing at end of operation)
PRE-OP	pre-operative (washing at start of operation)
RAF	gene encoding kinase regulated by *ras* (q.v.)
Ras	oncogene, first described in retrovirus induced rat sarcomas
R hemi	right hemicolectomy
rpm	revolutions per minute
RPMI 1640	Roswell Park Memorial Institute tissue culture medium 1640
RT-PCR	reverse transcriptase polymerase chain reaction
SB	small bowel
T	tumour (in TNM (*q.v.*) staging)
T4 ligase	DNA-ligating enzyme encoded by T4 bacteriophage
Taq	DNA polymerase encoded by *Thermophilus aquaticus*
TBE	Tris-borate-EDTA (*q.v.*)
Tcf	T cell factor
TEMED	N,N,N',N'-Tetramethylethylenediamine
TGFBR	gene encoding transforming growth factor-β receptor
T_m	"melting point" of double-stranded DNA
TME	total mesorectal excision
TNM	tumour, nodes, metastases staging system
TP53	Tumour suppressor gene encoding p53 (q.v.)
U	units
UICC	Union International Contre le Cancer
V	volts
v/v	volume per volume
WBC	white blood cells
Wnt	"wingless" gene first characterised in *Drosophila melanogaster*

CHAPTER 1: INTRODUCTION

1.1 Colorectal cancer

Colorectal cancer is a major health problem throughout the developed world. It is the second commonest cause of cancer death in the UK, and the leading cause of cancer death among non-smokers. In 1998, over 35,000 new cases were diagnosed, and in 2001 there were 16,000 deaths (Cancer Research UK website). In most cases, no obvious individual risk factors can be identified, although about 5% have a clear inherited basis (see section 1.6.1). A small proportion of patients also develop tumours on a background of chronic inflammation over many years, such as those suffering from ulcerative colitis. The commonest risk factor is increasing age, with a peak incidence in the eighth decade. On a population level, obesity and a diet high in fats and low in fruit and vegetables gives an increased risk of disease.

Patients present with a variety of symptoms including alteration of bowel habit, overt rectal bleeding, anaemia due to chronic blood loss, abdominal pain and symptoms of more advanced disease such as weight loss. Surgery is the major form of treatment for colorectal cancer, and most cures are achieved by surgery. The nature of the surgery performed depends on the site of the primary tumour, but normally involves removing the tumour, with a margin of healthy bowel, and the lymph nodes along the blood vessels supplying the affected part of the bowel. Some patients benefit from additional radio- and/or chemotherapy, which may be given before or after operation depending on the site and extent of the tumour (see section 1.2.1.2). Surgery may also be used to attempt cure of some patients with metastatic or recurrent disease (particularly in the liver), and palliative chemotherapy is widely used.

The overall five-year survival rate remains at about 40% in the UK. Almost 25% of patients present with disease that is already incurable, having spread to the liver or other metastatic sites (Colonna *et al.*, 2001; Newland *et al.*, 1987). About one third of patients who have undergone surgery which removes all visible tumour, later succumb to their

disease. Many of these can be predicted from the findings at operation, or the histopathologist's report, but a significant proportion occur without obvious risk factors.

Although advances in surgical technique and adjuvant treatment are producing some benefits in survival, probably the best hope for improving the outcome for patients with colorectal cancer lies in diagnosing the disease at an earlier stage. This may be achieved, in part, by greater public awareness of the disease and its symptoms. Because colorectal cancer has a long and well-characterised pre-malignant phase (section 1.6.2), it may prove a good candidate for a programme of screening, and the possibility of this is being examined currently.

Despite screening, many patients are likely to continue to present with established cancer. This group of patients will benefit from accurate assessment of the extent of their disease. Much can be done using current staging systems (see section 1.2), but further information is likely to become available in future from the additional use of techniques to detect disseminated colorectal cancer cells using molecular markers. This additional information will allow targeted follow up and adjuvant treatment, and may thus help to improve survival.

This thesis is concerned with the detection of such disseminated tumour cells in the peritoneal cavity of colorectal cancer patients around the time of surgery. In this introduction, I will describe current systems in use to predict recurrence, how and where recurrent colorectal cancer occurs, how local recurrence may be prevented, predicted, recognised and treated, strategies for improving outcomes in colorectal cancer, and the relevant molecular biology of colorectal cancer, which aids in understanding and possible implementation of these improvements.

1.2 Prediction of outcomes in colorectal cancer: rationale for staging and current practice

Patients with colorectal cancer form a heterogeneous group, with tumours with a variety of characteristics, and with a range of possible outcomes. There is a long history of

attempts to classify colorectal tumours into groups, or stages, according to their histological features. The reasons for such classification are discussed below.

1.2.1 Rationale for staging in colorectal cancer

1.2.1.1 Prognostication

Giving patients information about their disease is an important part of providing care for all patients, and particularly those with cancer. It has been shown that patients want information about their illness, and that providing such information can improve both physical and psychological outcomes. After the initial shock of a diagnosis of malignancy and the need for major surgery, the detailed histology report allows for reassurance that the surgery was necessary (especially if the pre-operative diagnosis was based on imaging rather than histological proof) and permits more personalised information to be given.

Patients with early tumours (e.g. Dukes' stage A, see below) can be reassured that a cure is almost certain after surgery alone. Those with more advanced disease can be advised about the probability of cure or of recurrent disease. The risks and benefits of additional treatment and the need for more rigorous follow-up (see below) can be discussed.

1.2.1.2 Selection of Patients for Adjuvant Treatment

Radiotherapy has been shown to be beneficial in reducing the local recurrence rate after surgery for rectal cancer, and also increased five-year survival in one study (1996). Radiotherapy is not routinely used in colon cancer management. In Europe and the UK, radiotherapy for rectal cancer is usually given pre-operatively (as in the Swedish study), as either short course treatment, over five days immediately prior to surgery, or as a long course, over several weeks, usually in combination with chemotherapy. Long course treatment is used to reduce tumours in size, and to attempt to make fixed or inoperable tumours amenable to surgery. Pre-operative radiotherapy is generally better tolerated than post-operative treatment, with fewer withdrawals due to side effects (Ooi *et al.*, 1999). The use of imaging investigations pre-operatively to assess tumour stage is important in selecting patients for radiotherapy, and attempting to reduce the morbidity caused by

unnecessary treatment. Imaging investigations are not completely accurate, however, and the histology report gives essential information about the circumferential resection margin. If this is involved with tumour, or if the tumour extends to within 1mm of the margin (considered as an involved margin, Royal College of Pathologists, minimum data set for colorectal cancer) and the patient has not already received radiotherapy, then post-operative treatment will be recommended. Accurate staging of the primary tumour in rectal cancer is therefore of particular importance.

The role of chemotherapy in colorectal cancer has expanded rapidly in recent years, following a number of large, multi-centre trials, which demonstrated a survival advantage for patients with Dukes' stage C tumours (1995). Although having had all macroscopic tumour removed by surgery, and having no evidence of metastatic disease at the time of surgery (other than in lymph nodes removed with the specimen), about two-thirds of these patients will die from recurrent disease. Often, this will be disseminated disease, with the liver the most common site, due to spread through the portal circulation. The rationale for the use of adjuvant chemotherapy in these patients is to destroy any residual cancer cells at a time when their numbers are least, i.e. soon after surgery. The absolute survival advantage for patients with Dukes' stage C colon tumours who receive adjuvant chemotherapy is about 5%. Assigning patients accurately to Dukes' stage C has therefore become increasingly important, so that potentially beneficial treatment is not withheld. Currently, Dukes' B patients are not routinely given chemotherapy, except as part of a clinical trial, because the benefits of treatment are insufficient to justify the risks and costs.

1.2.1.3 Audit and Quality Control

Surgeons are increasingly required to demonstrate that their care is of an acceptable standard. The surgeon has been shown to be responsible for considerable variability in post-operative morbidity and mortality (McArdle and Hole, 1991). Measures that can be used in auditing quality of care for colorectal cancer patients include operative morbidity and mortality rates, local recurrence rate, survival rates at various time points and the rate of permanent stoma formation. To allow these to be compared between institutions or

surgeons in any meaningful way it is necessary to allow for case mix, the differences between the groups of patients treated. One major influence on outcome is stage of disease at presentation, and therefore it is important that this is assessed and recorded in a standardised format to allow comparisons of similar patients to be made.

Accurate pathology reporting may also be useful in the assessment of the quality of surgery, especially for rectal cancer (Birbeck *et al.*, 2002; Nagtegaal *et al.*, 2002). Involvement of the circumferential resection margin after rectal cancer surgery has been shown to be associated with an increased risk of local recurrence (Quirke *et al.*, 1986) and can be used as a tool to compare surgeons. Accurate pathological reporting has been important in trials designed to assess the benefits of the introduction of new surgical techniques, such as total mesorectal excision (Martling *et al.*, 2000).

1.2.2 Current Staging Systems

A large number of different systems for staging colorectal cancer are in use around the world, and even at times within single institutions. This variety suggests that no single system is ideal for defining groups of patients with incurable disease, those who have been cured by surgery, and those who may benefit from adjuvant treatment. Many of the staging systems are based on that originally defined by Dukes, although sometimes with considerable modification, or even misunderstandings of his original definitions. The TNM classification is also in widespread use, and these two systems are discussed below.

1.2.2.1 Dukes' Staging

Dukes originally described his classification system for operable rectal cancers (Dukes, 1932), although he did suggest that it was applicable to all intestinal cancers, and it is now commonly used for tumours of the whole colorectum. This classification divides patients into prognostic groups based on the depth of involvement of the bowel wall and the presence or absence of lymph node metastases. The original classification included just three categories, with stage A tumours limited to the bowel wall, stage B tumours

having spread in direct continuity into extra-rectal tissues, but without involved lymph nodes, and stage C being any tumour with regional lymph node involvement.

Dukes' described the three groups as being different in survival at one, two and three years, different in distribution of histological grade (with higher grade tumours being of more advanced stage) and with higher operative mortality with increasing stage. Even 70 years ago, he suggested that the classification would 'serve to inform the surgeon as to whether or not he is getting the best results with a particular type of case'.

This classification has been revised a number of times and several different versions are in common use today. Patients with distant metastases are said to be stage D. Dukes himself subdivided stage C, with stage C2 corresponding to involvement of the apical lymph node (the node nearest to the tie on the main vascular pedicle of the specimen) and stage C1 any other nodal involvement.

Stage B has also been subdivided (Astler, 1954) with A tumours confined to the mucosa, B1 tumours extending into but not beyond the muscularis propria, and B2 including those tumours with direct spread beyond it. This so-called Astler-Coller modification of Dukes' staging included a similar division in stage C tumours, which additionally had involved lymph nodes. This classification has the advantage of sub-dividing the heterogeneous Dukes' stage B patients into two groups with different recurrence risks, according to the local extent of disease. However, it is not widely used in the UK, and is not used in the remainder of this thesis.

Dukes' staging has the advantages of simplicity, and of being widely understood. It does divide patients into groups with differing prognoses. For example, the five-year survival rates for patients in stages A, B and C are about 95%, 65% and 35% respectively. Dukes' classification is still used in the selection of patients for adjuvant chemotherapy (see above).

However, Dukes' staging alone does not always provide sufficient information. In particular, there is no emphasis on peritoneal or lateral margin involvement, and vascular invasion is not considered. As we will see, these features can give important prognostic information. Although many of those with Dukes' B tumours will be essentially cured by their surgery, the remaining third will suffer from recurrent disease. This may include those with advanced primary tumours but without nodal involvement. Because of these limitations, other staging systems are in use alongside or instead of Dukes' staging.

1.2.2.2 The TNM (Tumour, Nodes, Metastasis) Staging System

The TNM system of the Union Internationale Contre le Cancer (UICC) is used in the classification of many types of cancer throughout the world, including colorectal cancer. This system allocates a different number for each aspect of the cancer being reported. The classification for colorectal cancer is as follows (1997):

T stage- Primary tumour

TX: primary tumour cannot be assessed

T0: no evidence of primary tumour

Tis: intra-epithelial tumour only

T1: tumour invades submucosa only

T2: tumour extending into but not through the muscularis propria

T3: tumour extending through muscularis propria into subserosa or non-peritonealised pericolic or perirectal tissues.

T4: tumour directly invading adjacent organs, or perforating the visceral peritoneum.

N stage- regional lymph nodes

NX: regional lymph nodes cannot be assessed

N0: no regional lymph node metastases

N1: metastases in 1-3 regional lymph nodes

N2: metastases in 4 or more regional lymph nodes

M stage- distant metastases

MX: distant metastasis cannot be assessed

M0: no distant metastases

M1: distant metastases

The advantages of the TNM system are that it is widely understood across a large number of countries, allowing for international comparison. Its use for a variety of different cancers means that many clinicians are familiar with the system. The criteria for each stage are clearly defined and regularly revised to take account of new research and developments. The allocation of a separate number for each element allows, for example, a locally advanced tumour without nodal metastases to be classified as T4N0, giving a clearer picture of the problem than just labelling it as Dukes' stage B. It is also possible to use the same classification system pre-operatively, based on imaging findings.

One disadvantage of the TNM system is that it may be seen as too complex. Because each element of the disease is classified separately, the number of possible categories is large, and less easy to see at a glance than a simple letter. This has led to some groups subdividing the TNM classifications into 4 stages, which correspond to Dukes' stages A to D. This leads to the loss of many of the advantages of the TNM system. The lack of a specific classification for the involvement of the apical node (demonstrated to be an independent prognostic factor (Newland *et al.*, 1994)) could also be a weakness. However, the number of involved nodes is also a prognostic factor (Jass *et al.*, 1987).

Any attempts to improve the usefulness of staging rely on an understanding of the behaviour of colorectal cancer, particularly the methods and patterns of recurrence, and this topic is considered next.

1.3 Mechanisms and patterns of Recurrence in Colorectal Cancer

1.3.1 Mechanisms of recurrence

As with all solid tumours, colorectal cancer has four major modes of possible spread. These are:

1 Direct invasion. The tumour adheres to and then invades an adjacent organ, for example a loop of small bowel, in the case of a colonic tumour, or the vagina in the case of a rectal cancer.

2 Lymphatic invasion. Tumour cells are transported to local and then regional lymph nodes *via* lymphatic channels. This route is common in colorectal cancer, and is addressed in all the staging systems in common use (see section 1.2).

3 Haematogenous spread. Tumour cells gain access to the blood circulation either by invading capillary blood vessels locally or *via* the lymphatic system and are transported around the body to other organs, where some cells may escape from the circulation and form metastases. For colorectal tumours, these metastases commonly occur in the liver, the first capillary bed encountered by cells in the portal circulation.

4 Transcoelomic spread. This occurs when tumour cells are released into the peritoneal cavity. This may occur due to full thickness invasion of the bowel wall by tumour or by leakage of cells from cut lymphatic channels during surgery. The cells may establish metastases locally, near the site of the original tumour, or elsewhere on peritoneal surfaces or the ovaries.

All these forms of spread are common in colorectal cancer and give rise to the various patterns of recurrence discussed below.

Colorectal cancer has a considerably higher rate of local recurrence when compared with some other solid tumours such as breast cancer. This high rate of local recurrence is postulated to occur because of the release of emboli of tumour cells from cut lymphatics within the specimen during surgery (Sugarbaker, 1991). Tumour cells released into the peritoneal cavity during surgery are likely to be able to implant much more efficiently than those metastasising in the circulation are. Intact endothelium does not readily allow tumour cells to implant; however, raw surfaces, which occur following dissection, provide a more favourable environment. The tumour cells may be trapped in the fibrinous exudates formed as a response to surgery, and may be protected from host defences as a result. The later release of growth factors, during repair and remodelling, may increase the rate of tumour growth.

Recurrence of colorectal cancer at the anastomosis has received particular attention (Umpleby and Williamson, 1987). This is thought to result either from implantation of tumour cells or by metachronous carcinogenesis i.e. formation of a new tumour. If occurring as a result of implantation, the tumour cells may originate from outside the bowel, by mechanisms such as those described above, or rarely, from inside the bowel lumen. The fact that viable cells are shed within the bowel is illustrated by the occasional tumour arising in a fistula, fissure or haemorrhoidectomy wound from an unsuspected proximal tumour. The risk of anastomotic recurrence can be reduced by the use of cytocidal washouts of the bowel ends prior to formation of an anastomosis. Some later occurring anastomotic recurrences may be due to new tumours arising in mucosa that is vulnerable to carcinogenesis as part of a 'field change'.

1.3.2 Patterns of Recurrence in Colorectal Cancer

The literature on patterns of recurrence in colorectal cancer is somewhat confusing, with a large number of small series, and considerable variation in the definitions of local recurrence and distant metastases. For example, local recurrence is sometimes used to refer to only recurrence at the tumour bed or in the anastomosis, and in other papers also includes peritoneal seeding. Some authors use a broad classification of intra-abdominal recurrence, which includes the above categories as well as those with liver metastases. The rate of reporting of metastases at different sites will vary with the follow up investigations used. Another problem is that many of these series contain data on patients who had their surgery several decades ago, and who would not have had access to modern imaging, adjuvant treatment or surgical techniques. Some examples of reported recurrence rates at various sites are given below.

An American series of 322 patients, followed after curative resection for colorectal tumours (Brethauer, 2002) included 48 patients with recurrence (on whom data were available). Of these, 15 had only local recurrence (tumour bed or anastomosis), 26 distant metastases only, six had a combination and two had peritoneal seeding, i.e. multiple widespread tumour deposits throughout the peritoneal cavity (in combination with local or liver recurrence). The mean time to diagnosis of recurrence was 24 months.

Russell and co-workers studied a large group of patients with tumours proximal to the peritoneal reflection (Russell *et al.*, 1984). They initially described 245 patients who presented with disseminated disease. The liver was the only site of metastases in one third, and liver involvement was present in 80%. Other common types of metastases included unresectable proximal lymph nodes, direct invasion of a wide variety of other structures and peritoneal seeding, which was the only type of dissemination in 7.5% of this group. They followed a further 550 patients who had undergone surgery with curative intent, of whom one-third suffered recurrence, 72% within 2 years. Local recurrence (tumour bed, anastomosis, contiguous organs) occurred in 30%, and was the sole site of recurrence in 13%. Nodal involvement or peritoneal seeding was the sole site in 18% and some component of recurrence in almost 40%. 22% had liver metastases alone. The same group studied the results of post mortem examinations on 53 patients who died with evidence of recurrent colorectal cancer after previous apparently curative resection (Russell *et al.*, 1985). Ten patients had recurrence only in the operative bed or retroperitoneal nodes; 23 had disease confined to the abdomen, with 30 having widespread dissemination to sites including lung and brain.

In another paper focussing on colonic cancer (Willett *et al.*, 1984) over 500 patients underwent potentially curative surgery, and 31% suffered recurrence in some form. 19% suffered local recurrence, with only one third of these having local recurrence alone. This relatively small proportion may be due to the classification of peritoneal surfaces and proximal lymph node groups as distant metastatic sites.

An Austrian series (Schiessel *et al.*, 1986) reported local recurrences in 90 of 715 patients having radical surgery for colorectal cancer (13%). Three-quarters of these patients had local recurrence only (defined as tumour growth in the region of the primary operation). Only one sixth of recurrences occurred more than two years after surgery. They noted that local recurrence was significantly more likely after treatment for rectal rather than colonic cancer. Very similar results were noted in a group of 500 Italian patients (Stipa *et al.*, 1991). 31% of patients relapsed, with 13.6% having local recurrence. One quarter of

the patients with local recurrence also had distant metastases. Again, local failure was commoner after treatment for rectal cancer.

The impact of more modern imaging on detection of recurrence can be seen if these series are compared with that of (Cass et al., 1976). This older series found a similar overall recurrence rate of 37%, but described 27% of patients as having some component of local recurrence. Perhaps some of these patients also had disseminated disease, which would have been detectable with ultrasound or CT scanning. This is not an isolated series, with others also reporting higher rates of local failure (Chung *et al.*, 1983). Of the 29% who developed recurrent disease, isolated local failure was found in almost half, and 80% had some element of local recurrence.

In summary, the overall rate of recurrence in these series of patients undergoing surgery thought to be curative was 30-35%. The variable definition of local recurrence makes comparison of the series difficult, as does the exclusion of rectal tumours from some studies, but between 13% and 80% of those patients whose disease recurs have some component of local recurrence. Isolated local recurrence is seen in 6-50% of those who suffer recurrence (typically 10%).

1.4 Prevention, prediction, detection and treatment of local recurrence in colorectal cancer

1.4.1 Prevention of local recurrence

The problem of local recurrence in rectal cancer has received particular attention in recent years, with developments in surgical technique and the increasing use of radiotherapy. As we will see, the rate of recurrence, and particularly local recurrence, is significantly higher in those patients with rectal rather than colonic cancer. Although this could reflect differences in tumour biology, it is more likely that the surgical inaccessibility of the rectum is responsible for much of the difference. Initially the problem was thought to relate to inadequate length of bowel excision distal to the tumour. However, it has been demonstrated that tumour deposits are very rarely found within the bowel wall more than 2cm distal to the primary tumour. The work of Heald in

popularising the concept of total mesorectal excision (TME) and of Quirke, in emphasising the need for accurate pathological assessment of the lateral or circumferential resection margin has focussed attention in this area.

Even recent review articles quote rates of local recurrence in rectal cancer as being as high as 40 or 50%, with rates of 25% in the 'best centres'(Radice and Dozois, 2001; Temple and Saettler, 2000). Reports from single surgeons using TME have demonstrated local recurrence rates below 5% (MacFarlane *et al.*, 1993). The TME procedure involves careful dissection in the so-called 'holy plane' around the mesorectal fascia, along with preservation of the pelvic nerves. For tumours of the middle and lower thirds of the rectum the whole mesorectum is excised, down to the pelvic floor; for tumours of the upper third the mesorectum is divided at least five centimetres below the tumour. This policy results from the detection of tumour deposits up to 5cm distal to the tumour in the mesorectum, but not in the bowel wall. It has proved possible to transfer the benefits of TME to other centres, with appropriate training of surgeons (Martling *et al.*, 2000). This study reported the reduction in local recurrence rates, permanent stoma formation and mortality following the introduction of TME surgery in Stockholm.

Another mechanism for reducing local recurrence after rectal cancer surgery is the use of radiotherapy (1996). The rate of local recurrence was halved in this study, and a survival advantage was apparent. Radiotherapy has been considered further in section 1.2.1.2.

Adequate surgical technique is also likely to be beneficial in reducing the local recurrence rate in colonic tumours (Read *et al.*, 2002). In a study of over 300 patients, local control was achieved in 96% at 5 years with the use of careful anatomical resection and proximal ligation of blood vessels.

In patients with risk factors for local recurrence, such as perforation or invasion of adjacent organs, some authors advocate the use of chemotherapy within the peritoneal cavity (Sugarbaker, 1998). This can be used during surgery, when it is often heated to

enhance the cytotoxic effects, and also during the first few post-operative days. Full assessment of the role of this treatment awaits further research.

1.4.2 Prediction of local recurrence

Along with the interest in reducing local recurrence after rectal cancer surgery, there has been growing interest in the use of pathology to predict those at increased risk. Adequate assessment of the circumferential margin of the specimen requires specific preparation and examination techniques. Of the quarter of cases with involved margins in one study (Quirke *et al.*, 1986), 85% suffered local recurrence, compared with 3% in the group with clear margins. Circumferential margin involvement also reduces survival, and is suggested as a tool for surgical audit and the monitoring of surgical training programmes (Birbeck *et al.*, 2002).

Considering the prediction of the likelihood of local recurrence in all colorectal tumours, a number of authors have described a consistent set of risk factors. Those describing patterns of recurrence, plus others (Chung et al., 1983; Michelassi et al., 1990; Phillips et al., 1984) describe a greater risk of local recurrence with:

- Increasing stage of penetration of the bowel wall by primary tumour (advancing T stage)
- Advancing Dukes' stage
- Poorly differentiated tumours
- Tumours presenting with bowel obstruction
- Tumours clinically fixed to surrounding structures
- The presence of vascular or lymphatic invasion
- Rectal cancers compared with colonic cancers, and left rather than right colonic tumours.

Efforts to increase the rate of detection of cancer cells in the peritoneal cavity, as a marker for bowel wall penetration (and therefore a risk factor for local recurrence) are discussed in section 1.5.3.

1.4.3 Detection of local recurrence

Most current efforts to identify early those with local recurrence are based on clinical follow up, rather than any specific techniques. Some recent evidence, from a meta-analysis of randomised controlled trials (Renehan *et al.*, 2002), suggests that intensive surveillance of colorectal cancer patients may be beneficial. Those subjected to an intensive regime trying to detect extra-mural recurrent disease (by CT scanning and serial CEA measurements) had improved survival, with a risk ratio for mortality of 0.73 (95% confidence interval 0.60 to 0.89). The most notable difference was in the proportion of patients diagnosed with local recurrence at an isolated, and therefore potentially treatable stage (15% in the group with intensive surveillance, versus 9% in the group with routine follow up, risk ratio 1.61). These results contrast with previous work, which has tended to dismiss the value of intensive follow up (Kievit, 2002).

One problem is the difficulty of distinguishing recurrence from scar tissue using CT scanning or other conventional imaging modalities. One possibility to improve the specificity of follow up is the use of positron emission tomography (PET). This technique relies on the increased metabolic activity of malignant tissue, compared with normal tissue. It has been shown to be 100% accurate (in 23 patients with 25 lesions) in distinguishing granulation tissue and recurrence in the pelvis after rectal cancer surgery (Takeuchi *et al.*, 1999) and is said to be more sensitive and specific than CT in imaging local recurrence (Arulampalam *et al.*, 2001). PET is not currently easily available in many UK centres, and therefore cannot be used routinely in all patients. It may be helpful to identify a group who are likely to suffer from local recurrence so that best use can be made of the facilities available.

1.4.4 Treatment of local recurrence

If local recurrence can be identified while it is still localised, then potentially curative treatment is possible in some patients. If such treatment is feasible (usually involving major surgery, often with excision of adjacent involved organs) then the 5 year survival rate is likely to be around 40% (Stipa *et al.*, 1991). However, in this study, only 8% of

those with local recurrence were suitable for such treatment, despite being involved in a regular follow up programme, aimed at early detection of recurrence.

The proportion of patients amenable to radical surgery is likely to be higher if intensive follow up means that many local recurrences are detected before they become symptomatic (Schiessel *et al.*, 1986). This group found that 42% of patients were able to undergo potentially curative procedures. For those patients where radical surgery is not appropriate, palliative surgery may help to relieve symptoms. A few long-term survivors may be found after palliative radio- or chemotherapy.

In a group of patients coming to surgery for local recurrence after primary treatment of rectal cancer, 29% were operated on with curative intent (Suzuki *et al.*, 1996). This surgery sometimes involved partial excision of the sacrum. With the addition of chemo- and/or radiotherapy in some cases, the median survival of the 'curatively' treated group was 44 months, with 38% disease free after 3 years.

If re-resection with curative intent is possible, as it was in 24% of patients in a large Canadian series (Obrand and Gordon, 1997), then survival is prolonged compared to those undergoing palliative or no treatment. In this study, 47% of the group treated for cure were long term survivors.

These studies show that treatment is possible for a proportion of patients who develop local recurrence of their colorectal cancers. If all detectable disease can be removed, than 5 year survival figures are in the order of 40%. The challenge is to detect patients with local recurrence at a stage when such treatment is possible. Intensive follow up and PET scanning may prove to be of benefit. Additional techniques, focussing on the detection of peritoneal involvement with cancer, as a marker for likely local recurrence, are discussed below.

1.5 Improving outcomes in colorectal cancer

1.5.1 Screening and early diagnosis

As we will see, colorectal cancer has a long and well-characterised pre-malignant phase (adenoma). The most attractive way to reduce deaths from colorectal cancer is to reduce the number of cancers developing, by detection and removal of adenomas. American guidelines suggest that healthy adults should enter a programme of regular colonoscopy from the age of fifty. Mass colonoscopy is not considered feasible in the UK at present, but a programme of faecal occult blood testing, with colonoscopy for those with positive results, has recently been tested in two pilot areas, with the English pilot site being Coventry and Warwickshire. The result of this, regarding both benefits and costs is currently being analysed.

Another possible means of improving outcome in colorectal cancer is to ensure that cancers are detected at an early stage. This may be achieved in part by screening, but would also be helped by greater public awareness of the symptoms of colorectal cancer. Prompt access to the necessary assessment and investigation should also be available.

For the future, the use of chemopreventive strategies using drugs such as non-steroidal anti-inflammatories may become an option.

1.5.2 Techniques Used to Improve Staging of Colorectal Cancer

As we have seen in section 1.2.2, neither of the commonly used systems for staging colorectal cancer is ideal. A number of alternatives have been suggested, which attempt to address some of their weaknesses. Some of these have entered routine practice in particular regions or countries, while others currently remain as research tools only.

The Australian Clinico-Pathological staging system (Newland *et al.*, 1987) consists of four main stages divided into substages. The main features of this staging system are the subdivision of stage B (tumour invasion beyond the muscularis propria, without lymph node metastases) into B1 and B2 on the basis of free mesothelial surface invasion, and the inclusion of stage D (incurable disease due to distant metastases), which can be

defined either histologically or clinically (D1 or D2). This system has been used in a series of over 1000 cases with 14-year follow up, and has been shown to be valid, with a significant reduction in survival with free mesothelial surface invasion or apical lymph node involvement.

Close attention has also been paid to the relationship between the tumour and the peritoneal surface in the concept of local peritoneal involvement or LPI (Shepherd *et al.*, 1997). LPI was divided into four categories, with 58% of patients falling into the less favourable groups 3 and 4. These patients had a significantly poorer prognosis, even after curative surgery, and all but one of the 46 patients with intra-peritoneal recurrence or persistence of disease were in these groups.

Standardised pathology reporting has been introduced for colorectal cancer specimens. This specifies a minimum data set to be collected for each sample submitted. It includes both Dukes' and TNM stage to allow it to be understood readily by all members of the multi-disciplinary team. Use of this standardised format allows easier collection of data within an institution, and submission of data to regional or national databases, allowing for audit of workload and results.

One aspect emphasised is the accurate recording of the number of lymph nodes examined from each specimen. Under-staging of colorectal cancer is more likely to occur if only a few nodes are examined, although the exact number required for accuracy is unclear, having been estimated at between 6 and 17 per patient (Cserni *et al.*, 2002). This group found that survival increased with the number of nodes retrieved, in a group of patients with T3N0 tumours, suggesting under-staging in those with fewer nodes retrieved. The TNM classification suggests that 'histological examination of a regional lymphadenectomy specimen will ordinarily include 12 or more lymph nodes'. The number of nodes recovered can be increased by clearing fat from the specimen, and this allows some patients to be reclassified from Dukes' B to Dukes' C (Koren *et al.*, 1997; Scott *et al.*, 1994).

Other workers have suggested means by which the nodes obtained can be examined more thoroughly. Current practice involves a single section from each node being examined after haematoxylin and eosin staining. Research has suggested that using multiple sections from each node may increase the detection of metastatic disease, but this causes considerable extra work for the histopathologist (van Wyk *et al.*, 2000). The use of immunochemical staining also increases the yield of positive results (Isaka *et al.*, 1999). The concept of the sentinel lymph node (the first node draining the tumour) has been popularised in breast cancer and malignant melanoma, as a means of reducing the morbidity of lymph node dissection. Although this aspect is not likely to be so important in colorectal cancer, where most of the morbidity is due to the extent of the incision and the risk of anastomotic failure, there is evidence that focussing on one node for more detailed histological examination may be helpful.(Bilchik *et al.*, 2001; Wood *et al.*, 2002)

1.5.3 Techniques for the Detection of Peritoneal Involvement

We have seen that involvement of the peritoneal (serosal) surface with tumor cells is an adverse prognostic feature, and is particularly associated with local recurrence of disease. Because of this, interest in the detection of cancer cells on the peritoneal surface of colorectal tumours or in washings taken from the peritoneal cavity during surgery is longstanding.

Initially the washings were analysed by cytology alone, with Giemsa or Papanicolou staining. Moore found positive results in 18 of 77 patients with 'curable' disease and 20 of 43 with incurable tumours (Moore, 1961). He did note that there were problems with false positive results due to the difficulty in distinguishing altered mesothelial cells from malignant cells based on morphology alone.

Because of these problems, a number of groups explored the use of monoclonal antibodies, for example to CEA or mucin, to improve the specificity of detection of malignant cells in peritoneal washings. Some of the results were disappointing, with poor correlation between results with monoclonal antibodies and conventional cytology (Ambrose *et al.*, 1989), or a very low detection rate in tumours without widespread

dissemination (Murphy *et al.*, 1993). Others were more successful, detecting cancer cells in a quarter of patients, with the rate increasing with advancing grade and stage (Juhl *et al.*, 1994). More recently, the use of a panel of antibodies allowed the detection of malignant cells in peritoneal washings in 34 of 109 patients with colorectal cancer (Schott *et al.*, 1998). Those with positive results had significantly poorer 4 year survival overall, with the same pattern being seen across all stages of disease.

The detection of serosal surface involvement by tumour cells may be improved by the scraping or imprinting of cells onto a slide, immediately after removal of the tumour specimen. Scraping the slide over the tumour and examining the result by conventional cytology showed serosal involvement by tumour in 23% (Zeng *et al.*, 1992) Imprint cytology detected 6 of 7 patients with histological serosal involvement, and detected a further 5 cases (out of 20) which were not thought to have such involvement (Murphy *et al.*, 1994)

1.5.4 Molecular Biological Techniques in Colorectal Cancer

The powerful tools of molecular biology have been applied to patients with colorectal cancer in recent years. This research has been driven by a desire to improve on the accuracy of currently available staging techniques. The aim of using such techniques is to detect very small numbers of cancer cells against a background of very large numbers of normal cells, with greater sensitivity than can be achieved with conventional histopathology, or even immunochemistry. It is believed that cells detected in this way act as markers for the likelihood of recurrence, and that detecting them is therefore important for all the reasons described above, particularly selection for chemotherapy. Some groups have also used molecular features of the primary tumours themselves in an attempt to predict prognosis.

1.5.4.1 Detection of minimal residual disease

Much of the work has concentrated on studying lymph nodes, as involvement of these with tumour is already known to be a prognostic factor. The techniques have also been applied to the blood (both in the draining mesenteric veins and in the general circulation)

to look for evidence of cells that may be in the process of metastasising. Although bony metastases are rare in colorectal cancer, it is believed that the bone marrow could act as a reservoir of cancer cells which later cause disease, and therefore malignant cells have been sought in this compartment. Relatively little work has been published on the peritoneal cavity as a possible site for residual cancer cells after surgery. This is in contrast with the situation in upper gastrointestinal tumours, where the peritoneal cavity is more commonly studied.

Although many researchers have published findings in this field, with evidence that the detection of particular tumour associated DNA or RNA is associated with poorer prognosis, this work has not been standardised. Reported rates of detection vary widely, as do rates of false positives with particular markers. For this reason the results of molecular tests are not currently incorporated in the TNM classification (Hermanek, 1999). It is suggested that they should be recorded clearly, with the precise technique noted, to allow comparisons between series. If some groups incorporate the results of molecular tests into the TNM classification, then stage migration may occur, hampering comparison of results. Cells detected by molecular techniques should be described as isolated tumour cells, rather than micrometastases, because the latter term has a specific definition implying arrest and implantation of tumour cells.

Although the ability to detect very small numbers of residual malignant cells after surgery seems very attractive, and would intuitively seem to suggest that such patients should be offered chemotherapy, it is important to consider two points. Firstly, the vast majority of those who are cured of colorectal cancer are cured by surgery, with those treated with adjuvant chemotherapy only gaining a small additional benefit. Unless or until more effective adjuvant treatment is available, the benefits of treating those with minimal residual disease, diagnosed using molecular techniques, is likely to be small. Secondly, until more prolonged follow up is available, the appropriate degree of sensitivity for molecular tests is not clear. It is technically satisfying to be able to detect one malignant cell in a million or even ten million normal cells, but data are lacking on whether such cells are of any clinical significance (Sidransky, 1997). Tests that are only

able to detect one malignant cell in hundreds or thousands of normal cells may in fact be more appropriate in giving relevant prognostic information.

The molecular biological techniques commonly used for detection of isolated tumour cells in colorectal cancer can be divided into two broad groups. The first and more commonly used group includes those techniques based on the detection of RNA, and the second group consists of DNA based techniques. The advantages and disadvantages of each, together with some examples of the results obtained, are discussed below.

1.5.4.2 RNA based techniques

Techniques based on the reverse transcriptase polymerase chain reaction (RT-PCR) involve the extraction of RNA from the tissue being studied, reverse transcription of this to form complementary cDNA and then amplification of this DNA with a polymerase chain reaction. These techniques have the major advantage that they can be applied to samples from all colorectal cancer patients, rather than those where specific mutations in the primary tumour have been characterised. These techniques rely on the detection of epithelial specific mRNA in an environment where epithelial cells would not normally be present, such as the bone marrow or the peripheral blood. It is assumed that all cells derived from epithelial tumours will express such mRNA, and this is significantly easier than attempting to look for particular DNA mutations, which vary from tumour to tumour. The relative fragility of RNA compared with DNA may also be an advantage because mRNA is most likely to be derived from a living cell, which could have gone on to form a metastasis, and not to be present freely in the circulation, or to come from a dead or damaged cell.

The major disadvantage of RT-PCR techniques is the very high rate of false positive results. All the suggested mRNA markers, which are used to detect isolated tumour cells, have produced false positive results under certain circumstances. A number of reasons have been suggested for these false positive results. It is possible that supposedly epithelial-specific mRNAs are expressed at low levels in mesenchymal tissue such as bone marrow (so-called illegitimate expression (Zippelius *et al.*, 1997)). If sufficient

amplification of the transcribed DNA was performed then this could result in a false positive result. Inflammation or hormonal effects may up regulate target mRNA expression. Secondly, primers may anneal to and amplify pseudogenes. These are non-functional DNA sequences with a high degree of homology to the functional genes under study. Finally, epithelial cells may be introduced into the sample. This could occur during the passage of the venepuncture needle through the skin, and would seem likely to be a problem with samples collected from the peritoneal cavity during surgery, when skin and bowel are divided. False negative results may also be a problem because mRNA expressed in the primary tumour may be down regulated or lost in metastatic cells, or RNA may be degraded during processing (Vogel and Kalthoff, 2001).

Despite these problems, many groups have used RT-PCR to look for cells dispersed from colorectal tumours. For example, the proportion of patients with involved lymph nodes is increased if RT-PCR is added to conventional histology or immunochemistry. At least two studies suggest that these patients have a poorer prognosis than those with no nodal involvement on molecular testing, but most studies are small, and a large variety of different mRNAs have been studied. A large number of studies have looked for circulating colorectal cancer cells with RT-PCR. Most of these have used carcinoembryonic antigen (CEA) or one of the cytokeratins (CK) as the epithelial-specific mRNA to be sought. Many of the studies have demonstrated false positive results in healthy volunteers, limiting the usefulness of their results. However, most workers have concluded that there is an association between the presence of circulating tumour cells detected by RT-PCR and poor outcome (reviewed in(Tsavellas et al., 2001). Fewer groups have examined the bone marrow of colorectal cancer patients, but the same problems of illegitimate gene expression and pseudogenes have caused problems. CK20 is probably the marker giving the fewest false positive results. Although some studies have shown an adverse effect on outcome in those patients with colorectal cancer cells detected in this way, it is not clear whether this adds anything to standard histological assessment.

RT-PCR can be used in a quantitative fashion, with equipment such as the "Light Cycler". This relies on the generation of fluorescent PCR products, using specially designed primers, or the addition of a double-stranded DNA specific fluorescent dye, and the amount of product generated is recorded after each amplification cycle. An appropriate rate of increase of product formation can be defined, which may allow false positives from very low-level illegitimate expression of a particular mRNA to be reduced. This technique was used, in one study, to detect mRNA for CK20 and CEA in the peritoneum of colorectal cancer patients, and allowed the selection of a group of 11 patients (out of 39) who had poorer disease free and overall survival. In this study, examining peritoneal washings proved a more reliable prognostic factor than the detection of malignant cells in the blood (Guller *et al.*, 2002).

1.5.4.3 DNA based techniques

DNA based techniques rely on the polymerase chain reaction (PCR). This is used to amplify a specific portion of DNA millions of times over. When used to look for cancer cells bearing a specific mutation, either the PCR primers can be designed to produce a product only when mutant DNA is present (mutant-allele specific PCR, also called mutant-allele specific amplification or MASA), or primers can be used which amplify both wild type and mutant versions of an allele, with further techniques used to distinguish between the two types of product (see below).

The advantages of using DNA based techniques rather than RT-PCR are that DNA is more robust than RNA, reducing the risks of false negatives due to sample processing. The problem of illegitimate gene expression is removed by looking at the genetic code itself, rather than what has been transcribed from it. False positive results should not be caused by inflammation, which does not cause immediate DNA changes. These techniques are particularly important in an environment in which benign epithelial cells are likely to be present, where RT-PCR for epithelial specific-markers would be unhelpful, and this includes the peritoneal cavity around the time of surgery.

There are also major disadvantages of using DNA based techniques in colorectal cancer research. The most significant problem is the wide variety of different genetic changes seen in this disease. There is no single genetic mutation seen in all, or even most, colorectal tumours. Even when mutation of a particular gene occurs at high frequency, such as mutations of *TP53*, occurring in around 75% of cases, this consists of multiple different changes at different points in the gene. Because of this variability, it is not possible to use a single test when applying DNA-based tests in colorectal cancer. To be able to detect cells derived from even half of the tumours a panel of different tests is needed; our choice of mutations for study is discussed further in section 1.9. Indeed, some groups first determine the DNA sequence of commonly mutated genes by sequencing DNA from each tumour sample, and then design subsequent tests specifically for that tumour. Although the relative robustness of DNA can be advantageous, it may also be responsible for false positive results if DNA from lethally damaged cells can be detected.

Despite these difficulties, PCR-based techniques have been used to detect colorectal cancer cells in a number of different body compartments. MASA has been used to examine histologically negative lymph nodes, and the same mutation was found in the node as in the primary tumour in about half of cases. Patients in this group were more likely to relapse than those with MASA-negative nodes. Studying a combination of *K-ras* and *TP53* mutations meant that a mutation could be detected in 59% of the 120 patients studied (Hayashi *et al.*, 1995). *Ras* mutations were also detected in the blood in around one third of those with mutations in the primary tumour, and those with mutations had a poorer prognosis (Hardingham *et al.*, 1995).

One area where DNA-based techniques may prove particularly useful is in the detection of mutant DNA in the stool as a potential screening test. This may eventually prove more reliable than faecal occult blood testing in the detection of adenomas and early stage cancers, because enterocytes are shed continually from the bowel surface, whereas tumours may bleed intermittently or not at all. The major challenge has been in extracting amplifiable DNA from stool, which requires special techniques to overcome the presence

of PCR inhibitors. This problem has been overcome, and using a multi-target panel, including mutations of *K-ras*, *p53*, *APC* and *Bat-26* (a marker of microsatellite instability), mutant DNA could be detected in the stool of 90% of cancer patients and 80% of those with large adenomas (Ahlquist *et al.*, 2000).

Some examples of the different types of DNA based techniques are described below. A wide variety of different techniques is available for the detection of specific DNA mutations. The choice of appropriate technique depends on the exact nature of the mutation being studied, the type of samples available and local skills and experience.

1.5.4.3.1 *Mutant-Allele Specific PCR*

When a mutation occurring commonly in colorectal cancer has been characterised, it may be possible to design PCR primers, which will only amplify mutant and not wild type DNA. This may be straightforward in some types of mutation, for example a deletion of several bases, which causes considerable difference between wild type and mutant DNA. In the case of point mutations however, it may be very difficult to design a primer that will be specific enough to prevent amplification of wild type DNA when this is present in excess. The deliberate introduction of mismatches into the mutation specific primer, often in the penultimate base, may improve the specificity of priming (Newton *et al.*, 1989). Nested PCR (in which the whole area of interest is first amplified, and then mutation specific primers used for a second round of PCR) may increase the sensitivity of these tests (Iinuma *et al.*, 2000). Other workers have used a blocking oligonucleotide, complementary to the wild type sequence, and having a 3' chain terminator, to improve the specificity of the reaction (McKinzie and Parsons, 2002).

1.5.4.3.2 *Distinguishing PCR Products from Mutant and Wild Type DNA*

An alternative approach in the DNA based detection of small numbers of malignant cells is to use PCR to amplify the relevant gene in both normal and wild type variants, i.e. using primers which do not cross the mutation site. It is then necessary to use further tests to distinguish between the types of product generated.

Many of these tests rely on the mutant DNA having a different secondary structure, or being able to take up such a structure. This difference in structure may then cause different behaviour of the DNA during gel electrophoresis, and samples with this difference can then be assessed by direct sequencing. An example of such a technique is single strand conformation polymorphism, where PCR products are thermally denatured before electrophoresis, and aberrantly migrating (mutant) bands can be identified. Mutant DNA will produce a PCR product with different denaturation properties to those of wild type DNA. Gels with a gradient of temperature or concentration of denaturant will allow these products to be distinguished (Frayling, 2002).

Restriction enzymes are also useful in distinguishing PCR products derived from normal and mutant DNA. The presence of a mutation may either introduce a restriction site, allowing mutant DNA, but not wild type DNA to be cut by the relevant enzyme, or a restriction site may be removed by the mutation. It is also possible to design PCR primers that deliberately introduce a restriction site, where one would not normally be present. This site would only be present in either mutant or wild type PCR products, allowing them to be distinguished after restriction enzyme digestion (Jenkins *et al.*, 2002).

The Mismatch Ligation Assay (MLA) is another DNA based technique, useful for the detection of small numbers of malignant cells against a background of normal cells. The principle of this technique is described in detail in section 2.7.1. Briefly, it relies on the ability of T4 ligase to join matched, but not mismatched oligonucleotides, with the incorporation of a radiolabel. As described in section 1.9, we chose to use MLA for detection of several mutations in the colorectal tumours we studied. The experience of those who have this technique for similar work is therefore reviewed here.

Jen and co-workers (Jen *et al.*, 1994*b*) studied small dysplastic and non-dysplastic colorectal polyps and also microscopic aberrant crypt foci. They looked for mutations at codon 12A, 12B and 13 of *K-ras*. *Ras* mutations were found in 23% of hyperplastic polyps and 25% of dysplastic polyps. The authors also detected *APC* mutations using a different technique and suggested that an initial mutation in *APC* rather than *K-ras* was

more likely to cause small or microscopic colorectal lesions to progress to dysplasia, with later *ras* mutations playing a part in that progression.

Hibi and co-workers (Hibi *et al.*, 1998) utilised the MLA technique to look for mutant DNA in the serum of colorectal cancer patients. They felt that identification of this mutant DNA may help to assess the extent of disease at the time of diagnosis. *K-ras* mutations were sought in the primary tumour and serum samples using the technique described by Jen. They also studied *TP53* mutations by sequencing *TP53* in the primary tumours and designing specific oligonucleotides for each mutation detected. Of 44 tumours tested, 16 had *K-ras* mutations detectable by MLA, and in 3 of these patients the same mutation was detected in the serum. 33 tumours were tested for *TP53* mutations and these were detected in 10 tumours. Using the specially designed oligonucleotides, the same mutation was detected in the serum in 7 of the patients. Of interest was the fact that all 3 of the patients with *K-ras* mutations in their serum samples had Dukes' stage C or D tumours whereas 5 of the 7 with *TP53* mutations in the serum had Dukes' stage B disease.

MLA has also been used to study lymph nodes in patients undergoing liver resection for colorectal metastases (Sanchez-Cespedes *et al.*, 1999). They again detected *K-ras* and *TP53* mutations, first detecting the mutations in samples from the liver metastasis to be resected and then using MLA to study DNA from perihepatic nodes for the same mutations. Standard oligonucleotides were used for *K-ras* and those for the *TP53* mutations were designed specifically for each tumour. They found MLA to be capable of detecting 1 tumour cell in 1000 normal cells. Mutant DNA was detected by MLA in 12 of 51 nodes histologically and immunochemically negative for cancer cells. All histologically involved nodes were positive on MLA. Use of the molecular technique increased the number of patients with positive nodes from 5 to 8 out of 16, and these patients had a poor prognosis. The authors suggest that molecular techniques could be used to select a group of patients who will not benefit from aggressive surgery.

The possible use of these techniques as a screening tool rather than simply for prognostication in those known to have cancer has also been explored (Dong *et al.*, 2001). The feasibility of this was studied in 51 patients known to have colorectal cancer so that mutations found in the primary tumours could be compared with those detected in faecal DNA. MLA was chosen to look for *TP53* mutations, and in this case, a panel of oligonucleotides was designed for use in 3 different reactions, which together could detect 9 *TP53* mutations commonly found in colorectal cancer. *TP53* mutations were detected in the primary tumours using PCR and a ligation detection reaction and these were confirmed using MLA. The specific DNA mutations found in the stool samples matched those in the relevant tumour and *TP53* mutations were only found in the stool samples of those having mutations in their primary tumours. This technique may be useful for screening in the future if it can be confirmed that adenomas shed sufficient DNA and if an appropriate panel of mutations can be studied to allow detection of most adenomas.

MLA has also been used to study mutations in mitochondrial DNA in the serum of patients with colorectal cancer (Hibi *et al.*, 2001). Mutations in the primary were detected by sequencing and the oligonucleotides for MLA were designed individually for each tumour with a mutation. 7 out of 77 tumours had mitochondrial DNA mutations and the same mutation could be detected in the serum of 1 of these 7 patients.

Another group using MLA found *K-ras* mutations in 31 out of 90 patients, and 11 of these (35%) had mutant DNA detectable in their serum, including patients with Dukes' A tumours (Ito *et al.*, 2002).

1.5.5 Molecular markers of prognosis in colorectal cancer

In addition to the role of molecular biology in detecting minimal residual disease, the presence of particular mutations in a primary tumour may give information about the likely prognosis. Unfortunately, for many markers such as *TP53* mutation, the relationship with prognosis has not been straightforward, probably due to the complex range of different mutations in particular genes, and their interaction with other gene

products (Roth, 1999). However, a number of possible molecular markers of prognosis have been described.

Although *TP53* mutation itself is not a clear prognostic marker, such mutation appears to be associated with a poorer response to chemotherapy (Iacopetta, 2003). Cytoplasmic (but not isolated nuclear) staining for p53 was also associated with a poorer prognosis (Sun, 1992).

Various allelic losses have been demonstrated to occur commonly in colorectal cancer. Loss of 18q has been consistently associated with worse outcome. Indeed, in patients with Dukes' stage B tumours, those without loss of 18q had 93% 5-year survival, compared with 54% in those with loss of 18q (Jen *et al.*, 1994*a*).

As we will see, a subset of colorectal tumours arises by a pathway known as microsatellite instability. Although there is some conflicting data, it appears that this group of tumours does have a relatively favourable outcome (Houlston, 2001).

With the advance of 'gene chip' technology, capable of analysing the expression of thousands of genes from a particular tumour, it is likely that, in the future, it will be possible to group tumours with common features together, allowing targeted follow up and treatment (Walker and Quirke, 2002).

1.6 The genetic basis of Colorectal Cancer

In order to benefit from the potential of these molecular biological techniques to detect minimal residual disease, or to predict recurrence, it is necessary to understand the genetic basis of colorectal cancer.

As with all cancers, colorectal carcinogenesis involves alterations in DNA. Malignant cells arise from successive rounds of mutation, with clonal expansion of the cell with the newly mutated genotype, because it has acquired a growth advantage. These mutations

allow growth to occur beyond what is needed to maintain normal structure and function, and allow eventual migration of cells away from their original sites.

In this section, I will review the more important genetic changes that occur in colorectal carcinomas. Most of these tumours arise in patients with no particular genetic predisposition (sporadic cancers) and are due to acquired DNA damage, occurring at the somatic cell level, through exposure to environmental carcinogens etc. There are, however, two distinct types of colorectal cancer that show a clear-cut pattern of inheritance, and the germ line mutations found in these conditions can give us useful information about the genetic changes occurring in sporadic cases. Although similar genetic changes occur in both inherited and sporadic forms of colorectal cancer, the inheritance of one of the relevant mutations means that cancer occurs at a much higher frequency, and at a younger age, in those with one of the syndromes discussed below.

1.6.1 Inherited Predispositions to Colorectal Cancer

1.6.1.1 Familial Adenomatous Polyposis (FAP)

FAP is an autosomal dominantly inherited condition, which accounts for about 1% of colorectal cancers, and has a population incidence of about one in 8000. It is due to a germ-line mutation in the *adenomatous polyposis coli (APC)* gene, which as we will see is also important in sporadic colorectal cancer. The mutations in FAP commonly cause formation of an inactive truncated protein product. Patients develop large numbers of colorectal adenomas throughout their colons beginning in childhood or early adult life and, if untreated, colorectal cancer is inevitable at an early age. This type of mutation is also associated with other abnormalities such as duodenal tumours and abdominal desmoids and, in some cases, congenital hypertrophy of the retinal pigment epithelium (CHRPE). Individuals in affected families (identified on the basis of their history) are offered genetic screening (if the relevant mutation has been characterised). Those with the mutation, or where the mutation has not been characterised, require screening by endoscopy, when the typical multiple polyps can be seen. About one quarter of cases are due to a new mutation. Treatment is surgical, with excision of the at-risk mucosa by colectomy, often with formation of an ileal pouch. In members of families with an

attenuated form of the disease, with less than 100 adenomatous polyps, an ileo-rectal anastomosis with continued rectal surveillance may be used.

A number of other germ line *APC* mutations have been described. Unlike those seen in FAP, they do not cause protein truncation, but cause amino acid substitution. The effects of this are not as severe as FAP, but consist of multiple adenoma formation and familial clustering of colorectal cancer. These missense mutations appear to exert a mild dominant negative effect (Fearnhead *et al.*, 2001; Frayling *et al.*, 1998).

An animal model with an inherited *APC* mutation, the *min* mouse (multiple intestinal neoplasia) develops multiple small bowel polyps and tumours. These mice show an increased rate of production of new crypts in the intestinal mucosa by crypt fission. The molecular mechanisms by which *APC* mutations cause colorectal cancer are discussed below (section 1.6.3.1).

1.6.1.2 Hereditary Non-Polyposis Colorectal Cancer (HNPCC)

HNPCC is another autosomal dominantly inherited condition giving rise to colorectal and other malignancies (especially endometrial cancer). These patients were originally described as suffering from 'Cancer Family Syndrome' (Lynch *et al.*, 1966). HNPCC causes about 5% of colorectal cancers and has a population incidence of around 1 in 500. As the name implies, the increased risk of colorectal cancer does not occur due to widespread polyposis (unlike FAP). About 80% of patients with a germ line mutation of one of the genes affected in HNPCC develop colorectal cancer, and their tumours occur at a younger age than those with sporadic cancers (Lawes *et al.*, 2002). The tumours are more likely to occur on the right side of the colon (two thirds proximal to the splenic flexure), and are, on average, of more favourable Dukes' stage when compared with sporadic tumours. Some studies suggest that survival is also improved, compared to patients with sporadic cancer of the same stage. Patients with HNPCC do develop colonic adenomas, at a similar rate to the normal population, but these progress to cancers more rapidly than sporadically occurring adenomas, because the abnormality in HNPCC involves genes concerned with DNA repair (see below). This contrasts with FAP where

patients develop far more adenomas than the normal population but each progresses towards cancer at a normal rate (Kinzler and Vogelstein, 1996).

HNPCC affected families are identified using the Amsterdam criteria:
At least three affected relatives in at least two generations
One must be a first degree relative of the other two
Two successive generations must be affected
At least one person aged less than 50 at diagnosis of colorectal cancer
FAP should be excluded
Tumours must be verified pathologically.
These criteria have recently been broadened to include cancers of the endometrium, small bowel, ureter or renal pelvis (all of which are rare in the general population) as well as colorectal cancers.

Members of affected families are offered screening. In about 70% of cases a specific mismatch repair gene mutation can be identified, and molecular screening performed. Only those family members with the specific mismatch repair gene mutation are at increased risk of colorectal cancer and require follow-up. Those with the relevant mutation and those from families where a mutation cannot be identified require surveillance by colonoscopy.

1.6.2 Colorectal Carcinogenesis- The Adenoma→Carcinoma Sequence

The genetic events leading to colorectal cancer formation have been particularly well studied, when compared with other cancers. This has been possible because colorectal cancer is common, its development from normal tissues, through adenoma to carcinoma is well characterised, and because of the availability of material for study from various stages of development of tumours. This is due to the frequent and relatively non-invasive removal of pre-malignant lesions, and the routine excision of both primary colorectal tumours and metastatic lesions.

There is a large amount of both clinical data (from the study of patients with untreated polyps) and histopathological data (including the frequent occurrence of adenocarcinoma within an existing polyp) to suggest that the vast majority, if not all, colorectal cancers arise within pre-existing benign lesions. These adenomas probably arise in hyperproliferative areas of epithelium, and gradually increase in size, become more dysplastic and develop villous (rather than tubular) morphology. Although developing continuously, the stages of adenoma development can be conveniently considered as early (≤ 1cm diameter), intermediate (> 1cm diameter, without focus of cancer) and late (>1 cm and containing focus of cancer). It is proposed that step-wise progression through the stages occurs through the accumulation of multiple genetic changes.

Typical genetic changes at each stage involving well-known oncogenes and tumour suppressor genes have been described, and many of these are discussed in more detail below. For example, the earliest change normally involves chromosome 5q (the site of the *APC* gene), and this occurs with the change from normal to hyperproliferative epithelium. The *K-ras* and *BRAF* genes typically are mutated during the change from early to intermediate adenoma. The *TP53* gene is rarely mutated in adenomas, but its mutation is said to be characteristic of the change from adenoma to carcinoma. Further changes, involving cell adhesion molecules, and tissue proteases occur during the change to a metastatic phenotype. Although these mutations characteristically occur at different stages in the adenoma-carcinoma sequence, generally it is the accumulation of a number of different mutations rather than their exact sequence that is significant (Fearon and Vogelstein, 1990). It is likely that most tumours require four to six independent mutations in order to progress.

1.6.3 The Major Genes Subverted in Colorectal Cancer

Mutations occur in both oncogenes and tumour suppressor genes. Oncogenes are cellular genes, which are activated in carcinogenesis. These genes often control normal growth and development, and gain cancer-promoting potential by a change either in their coding region or in their regulation. The version of the gene present in normal cells is called the proto-oncogene. This can be mutated to produce a product that has increased activity,

more of a normal product (by modification of gene regulation) or a product with a prolonged active life. Any of these changes can increase the rate of replication of cancer cells. Mutations of oncogenes act in a dominant fashion, with only one copy of the gene needing to be mutated to have the cancer-promoting effect. An example of an oncogene commonly mutated in colorectal cancer is *K-ras*.

Tumour suppressor genes are genes that are inactivated in carcinogenesis. These genes typically function to control or restrain cell proliferation. Because these genes function in a recessive fashion, loss of function normally requires changes to both alleles of the gene in a particular cell (Knudson's two hit hypothesis (Knudson, 1971)). Often the first copy is inactivated by mutation (cf *APC* in FAP), with the second being destroyed by a deletion or insertion or by loss of heterozygosity. Occasionally, as with *TP53* mutations, the first allele to be mutated causes the formation of a protein which binds to and inhibits the function of the normal protein, and this mutation is described as a dominant negative. *APC* is an example of a suppressor gene commonly mutated in colorectal cancer. Further examples of oncogenes and tumour suppressor genes are given in table 1.1.

Oncogenes in colorectal cancer	Tumour suppressor genes in colorectal cancer
K-ras	*APC*
BRAF	*TP53*
CTNNB1	*hMLH1*
	hMSH2
	E-cadherin
	TGFBR

Table 1.1 Oncogenes and tumour suppressor genes in colorectal cancer

Since these genes are frequently altered in colorectal carcinoma, potentially they all can be used to identify cancer cells and so detect minimal residual disease. Hence, I shall discuss each in turn, particularly the ones studied in this thesis.

1.6.3.1 Somatic *APC* mutations

Acquired (somatic) mutations in the *APC* gene, as opposed to the germ line mutations occurring in FAP, occur in about 80% of cases of sporadic colorectal cancer. Mutations generally occur at an early stage of the adenoma-carcinoma sequence (see above), and have been noted in adenomas less than 5mm in diameter. *APC* is a large gene with over 8500 base pairs in 21 exons, although most mutations occur in exon 15, which contains 75% of the coding sequence. Almost all somatic *APC* mutations involve a truncated protein product with abnormal function.

APC is involved in a number of different cellular functions, abnormalities of which can contribute to malignant change in cells. Probably the most significant change occurs in the Wnt signalling pathway. This pathway plays a key role during normal animal development in a wide variety of organisms. In the absence of a Wnt signal, cytoplasmic β–catenin is destabilised by a complex including axin and the APC protein. When Wnt stimulation occurs, axin is removed from the β–catenin-binding complex, and β–catenin moves to the nucleus where it stimulates DNA transcription, acting as a co-activator of T cell factors (Tcf). Absence of functional APC, or impaired function due to protein truncation, leads to a reduction in the destabilisation of β–catenin. The *APC* mutations commonly found in colorectal tumours lead to a loss of β-catenin degradation sites (Bright-Thomas and Hargest, 2003). The build up of β–catenin leads to an activation of transcription of a number of genes including *c-myc* and *cyclinD1*, which is involved in controlling progress through the cell cycle. This is illustrated in figure 1.1. Many colorectal tumours that do not have *APC* mutations have other mutations affecting the Wnt signalling pathway (see section 1.6.3.2). Polyps form after *APC* mutation because activated Tcf causes cells to remain as crypt cells rather than differentiating and migrating up crypts to be shed (Bienz and Clevers, 2000).

APC has been described as having a 'gatekeeper' role in colorectal tumorigenesis. (Kinzler and Vogelstein, 1996) Gatekeeper genes are responsible for maintaining a balance between cell division and cell death. *APC* seems well suited to this role in the colon for a number of reasons. Firstly, *APC* mutations are detectable in the earliest

neoplastic lesions in the colon, very small adenomas. Secondly, patients with FAP do not develop a large range of other cancers, suggesting a specific role for *APC* in the large bowel. Thirdly, cells with *TP53* or *K-ras* mutations, but with wild type *APC*, do not progress to form clinically important tumours. Therefore, it appears that *APC* mutations are the exception to the rule that the order in which mutations occur in colorectal cancer is unimportant.

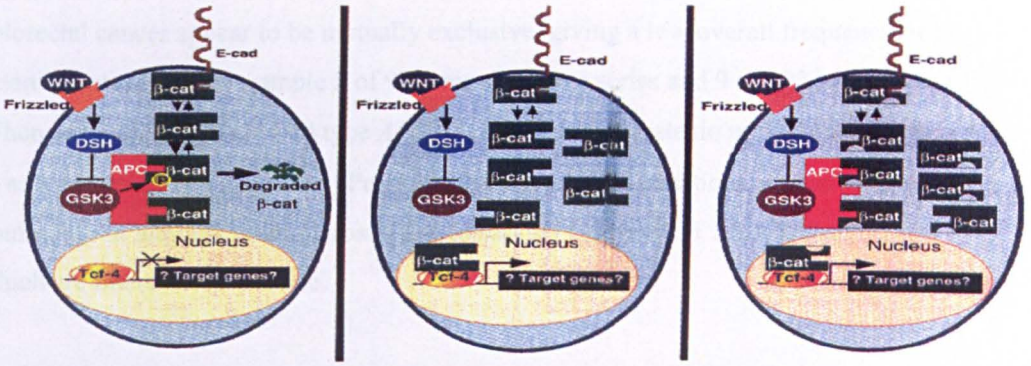


Figure 1.1 The function of APC and β-catenin in normal and mutant forms. The first panel illustrates the normal role of APC and glycogen synthase kinase 3 (GSK3) in targeting β-catenin for destruction. This pathway is inhibited by the Wnt signalling pathway acting *via* frizzled and dishevelled (DSH). The second panel illustrates the situation in the absence of functional APC; levels of β-catenin increase, and it acts as a cofactor for Tcf-4 causing transcription of target genes. The final panel shows the situation when β-catenin is mutated and cannot be targeted for destruction by APC. Again, transcription of target genes is increased. (E-cad= E-cadherin, see section 1.6.3.7) From Fearon, Human Cancer Syndromes: Clues to the origin and nature of cancer (1997).

A variety of other roles for APC have been described, and suggested as additional ways in which mutation of *APC* causes colorectal cancer. These include cell-cell adhesion, cytoskeletal stability, cell cycle regulation and apoptosis. All these complex interacting pathways are undergoing further research.

1.6.3.2 β-catenin

As discussed above, an alternative means by which the Wnt signalling cascade can be inappropriately activated in colorectal cancer, is through mutation of β-catenin (see

Figure 1.1). In colorectal tumours without *APC* mutations, point mutations have been found in the gene coding for β-catenin (*CTNNB1)*, which render it refractory to destruction by the APC complex (Bienz and Clevers, 2000). Experimental evidence has demonstrated colorectal cancer cell lines with wild type *APC*, but with constitutive activity in the β-catenin/Tcf transcription pathway, and these cell lines have been shown to have mutations in *CTNNB1* (Morin *et al.*, 1997). *APC* and β-catenin mutations in colorectal cancer appear to be mutually exclusive, giving a low overall frequency of β-catenin mutations, for example 2 of 92 tumours in one series and 9 of 202 in another. When only tumours with wild type *APC* are considered, β-catenin mutations were found in as many as 13 of 27 tumours (Polakis, 2000). β-catenin mutations are also seen more commonly in tumours with microsatellite instability (see section 1.6.3.3) than those which are microsatellite stable.

1.6.3.3 DNA mismatch repair genes

As described above, HNPCC occurs due to a germ line mutation of one of a number of DNA mismatch repair (MMR) genes such as *hMSH2* and *hMLH1*. The same mutations are also found in a proportion of sporadic tumours, in perhaps as many as 15% of cases. The role of the products of these genes is to check for errors occurring in DNA after replication, and to excise and replace mismatched bases. DNA repair is important because the genome may undergo tens of thousands of modifications per day, each of which could lead to residual damage. Absence or deficiency of MMR allows DNA mutations to accumulate, predisposing to cancer if present as a germ line mutation, or allowing tumour progression if occurring in a tumour. Mutation rates in tumours with MMR deficiency are 2 to 3 orders of magnitude higher than those seen in normal cells (Kinzler and Vogelstein, 1996).

DNA mismatch repair gene mutations can be detected by studying areas of DNA with repeated bases. These areas, known as microsatellites, will be unstable in cases of HNPCC, or in sporadic tumours with MMR mutations. Such tumours are said to have microsatellite instability (MSI). MSI is detected by using PCR to amplify five regions of DNA containing repeated sequences. MSI causes alteration in product length, or

appearance of a new band above or below the expected band. Tumours with MSI have abnormalities in 2 or more of the 5 markers. Some workers have described a group of tumours with low level MSI, and abnormality of only one marker, but these tumours do not share the characteristics of MSI tumours (Ward *et al.*, 2001). Sporadic tumours with MSI share a number of clinical and pathological characteristics when compared with microsatellite stable (MSS) tumours. They are more likely to be found in the right colon, to be poorly differentiated, of mucinous phenotype and to contain increased numbers of intraepithelial lymphocytes. *TP53* mutations (see section 1.6.3.6) are much less frequent in MSI tumours. MSI tumors have been reported to present at an earlier stage and have a better stage for stage prognosis than MSS tumours. Although these trends were confirmed in Ward's study, the differences did not reach statistical significance.

One gene commonly mutated in MSI tumours is that coding for the RII subunit of the TGF-β receptor (TGFBR). TGF-β normally acts in a growth-inhibitory fashion on colorectal epithelial cells. The affected gene contains a repeated polyadenine sequence, which is mutated in about 90% of MSI tumours, introducing premature stop codons and a truncated protein product (Markowitz, 2000).

A similar situation is seen with mutations in the gene for hTCF4, leading to uncontrolled transcription of its target genes (see above). Such mutations are found in 50% of MSI tumours, but rarely in microsatellite stable tumours (Bright-Thomas and Hargest, 2003).

1.6.3.4 *K-ras*

The products of the *ras* genes, $p21^{ras}$, play a key role in growth factor signalling pathways. *Ras* genes were named because they were first described in rat sarcoma viruses. There are several members of the *ras* family, with *K-ras* taking its prefix from the Kirsten murine sarcoma virus in which it was first described. *K-ras* codes for one of a series of proteins involved in transduction of signals between the cell membrane and the nucleus. Normal $p21^{ras}$ is active when bound to GTP, but not when bound to GDP. The intrinsic GTPase activity of $p21^{ras}$ hydrolyses GTP to GDP and controls $p21^{ras}$ activation.

K-ras is located on the cytoplasmic surface of the cell membrane, and receives input signals from several different pathways. Its most important effect in terms of proliferation is its attachment to RAF, which it recruits to the cell membrane. The serine kinase activity of RAF is normally inhibited, but binding to $p21^{ras}$ removes this inhibition. RAF is the first in a series of kinases, and activates subsequent members of the cascade, culminating in the phosphorylation and therefore activation of the transcription factor c-jun (Peyssonnaux and Eychene, 2001).

Mutations of *K-ras* occur in approximately 50% of colorectal cancers and large adenomas (Fearon and Vogelstein, 1990). These occur most commonly in codons 12 and 13, with codon 12B the most frequent site. Codon 12 represents part of the GTP binding region of the molecule, and mutation leads to decreased GTPase activity. Because the mutated $p21^{ras}$ is unable to hydrolyse GTP to GDP, it is constitutively activated and this leads to increased cell proliferation. An activating mutation such as this only needs to occur in one allele to have its effect on the cell. Although specific chemical mutagens have been described which cause *ras* mutations in rats, the cause of *K-ras* mutations in human colorectal cancer is not known. *Ras* pathways are attractive targets for drug development because they are affected in so many types of human cancer. To date, however, no successful drugs have been produced.

Mutations in *K-ras* may have effects on other pathways in colorectal tumorigenesis. For example, activation of the $p21^{ras}$ driven cascade (involving RAF and other enzymes) induces *Mdm2*, which causes degradation of p53. Thus, in tumours with *ras* mutations, the function of p53 can be impaired even in the absence of mutation. (Ries *et al.*, 2000). Activation of the *ras* pathway also causes release of β-catenin from its interaction with E-cadherin (see below), and stabilises free β-catenin within the cell (Bright-Thomas and Hargest, 2003).

1.6.3.5 *BRAF*

As I have indicated, the *raf* gene product transmits the growth factor signal from $p21^{ras}$ onwards through the other MAP kinases. Because of this association, and the

identification of a constitutively active form of *raf* as a viral oncogene, *raf* was chosen as one of the initial targets for the systematic search for mutations in human cancers that has developed from the Human Genome project. When a pathway includes one member known to be involved in many types of cancer, other members of the pathway are considered good targets for study. The knowledge of the DNA sequence for the whole human genome is giving the opportunity for researchers to seek out new cancer-associated mutations, to increase our knowledge of the biology of various cancers, and perhaps, eventually, provide new treatments. One of the first discoveries in this process has been the finding of mutations in the *BRAF* gene.

Workers at the Sanger Institute reported that *BRAF* somatic missense mutations were found in two-thirds of malignant melanomas, and a smaller proportion of a variety of other cancers.(Davies *et al.*, 2002) These mutations were initially detected using sequencing techniques, in a small panel of cancer cell lines where a lymphoblastoid cell line was available from the same patient for comparison. After establishing the presence of acquired mutations, a much larger panel of cell lines, and then the genomic DNA from a large number of primary human cancers was studied. In DNA from primary tumours, *BRAF* mutations were detected in 6 of 9 melanomas, 4 of 33 colorectal tumours and 5 of 35 ovarian tumours. A trend was noted for *BRAF* mutations to occur in tumour types known to have a greater proportion of cases with *ras* mutations.

Almost 90% of the mutations found in *BRAF* occurred within the activation domain, most commonly a single base substitution at codon 599. This mutant was shown to have ten times the basal kinase activity when compared with wild type BRAF. Normally, amino acids in the activation domain require phosphorylation, which occurs when the BRAF is recruited to the cell membrane by activated $p21^{ras}$. The BRAF mutations described in this activation segment appear to mimic phosphorylation and hence activate kinase activity in the absence of $p21^{ras}$ signalling. Cells with this mutation were able to continue proliferating in culture after the addition of a $p21^{ras}$ neutralising monoclonal antibody, indicating that this mutation uncouples the cell from its requirement for $p21^{ras}$ function.

Of interest is that none of the samples with codon 599 *BRAF* mutations had a *ras* mutation.

Further work has confirmed that *BRAF* mutations are found in almost 10% of colorectal tumours. (Rajagopalan *et al.*, 2002) This group studied 330 cases of colorectal neoplasia, finding *BRAF* mutations in 32, with 28 of these being a single T to A transversion at position 1796 (codon 599). In the same set of tumour samples, 169 had *ras* mutations, but no sample had both mutations ($p< 10^{-6}$). This could be expected since both function in the same pathway.

Both *Ras* and *BRAF* mutations occurred at all stages of cancer and were commoner in adenomas over 1cm diameter rather than smaller lesions. These results support the theory that Ras and BRAF mutations have similar effects in tumorigenesis, and occur at a similar stage in the adenoma- carcinoma sequence. An interesting difference between *Ras* and *BRAF* mutant tumours is that *BRAF* mutations are considerably more common in mismatch repair deficient tumours (i.e. tumours with MSI) compared to those with a proficient mismatch repair system. This pattern is not seen with *Ras* mutations, and is evidence that the specific mutation spectrum seen in a tumour depends on the underlying cause of genetic instability. (Kinzler and Vogelstein, 1996)

BRAF mutations in colorectal cancer have also been studied in samples obtained from patients in Hong Kong (Yuen *et al.*, 2002). In this group, 5.1% of colorectal cancers (11 of 215) had *BRAF* mutations, with only four of these being the T to A mutation at base 1796. There was no significant association of *BRAF* mutation with patient age or gender, or tumour site or differentiation. *BRAF* mutations were more common in tumours of less advanced Dukes' stage, but in this study, no association with MSI was shown. This group also studied colorectal adenomas and concluded that *BRAF* mutations occur in the adenoma stage, i.e. a similar stage to *Ras* mutations.

1.6.3.6 *TP53*

TP53 is described as the "Guardian of the Genome" because its major function appears to be in maintaining the genetic integrity of cells (Lane, 1992). This it does by controlling the apoptotic pathway to eliminate DNA-damaged cells. *TP53* is mutated or lost in over half of all human cancers. In colorectal cancer this figure is around 75%, with *TP53* mutations tending to occur at the stage of progression from a severely dysplastic adenoma to an invasive cancer (Navaratnam *et al.*, 1999).

The normal function of p53 is to block the progress of a cell into mitosis if DNA damage is present, enabling DNA repair to proceed. If the damage cannot be repaired then the cell will undergo apoptosis, so that DNA mutations are not passed on to its daughter cells.

Normal p53 activity is triggered by DNA damage, which is recognised by various molecules involved in cell cycle checkpoints. This leads to phosphorylation of p53, which changes it from a latent into an active form. The stability of p53 is also increased by being released from Mdm2. p53 increases the expression of proteins that block the cell cycle, allowing DNA repair to occur if possible (Houlston, 2001). If this is not possible, apoptosis begins to occur within a few hours of damage occurring, but this is absent in cells lacking p53.

If a cell has lost the function of p53, it is able to divide with damaged DNA, passing on mutations to the next generation of cells. A large number of different *TP53* mutations have been described, but all disrupt the ability of p53 to bind to DNA, and therefore to perform its function as 'guardian of the genome'. As stated previously, *TP53* mutations are an example of a dominant negative effect. p53 functions as a dimer, and when a heterodimer is formed with one mutant and one wild type p53 this is not able to bind DNA. This means that only one mutant allele is sufficient to impair p53 function (King).

Functioning p53 can also reduce the rate of cancer progression by killing cells that attempt to divide in oxygen-poor regions of tumours; mutant p53 cannot prevent such cell division. Tumours with *TP53* mutations may respond poorly to radio- and chemotherapy,

because these treatments depend, for at least part of their effect, on cell suicide in response to DNA damage. When *TP53* is mutated, such suicide is less likely to occur (King).

1.6.3.7 E-cadherin

E-cadherin (epithelial cadherin) is a member of a family of adhesion molecules concerned with intercellular contacts and the maintenance of normal tissue structure. The intracellular portion of E-cadherin binds to β-catenin. It has a dual role of promoting intercellular adhesion and inhibiting cell mobility (Navaratnam *et al.*, 1999). Loss of E-cadherin expression, which is found in about half of colorectal tumours, has been associated with tumour progression, invasion and metastasis. Experimental E-cadherin activation causes growth inhibition in tumour cell lines. Despite this important role, the loss of E-cadherin protein expression is not due to mutation or allele loss. Instead, hypermethylation of the promoter region of the gene has been shown to be responsible (Garinis *et al.*, 2002). Lack of E-cadherin may release β-catenin, increasing levels within the cell. If this cannot be broken down because of a lack of functioning APC, this may lead to increased activation of the Wnt signalling pathway. These interactions are illustrated in figure 1.2.

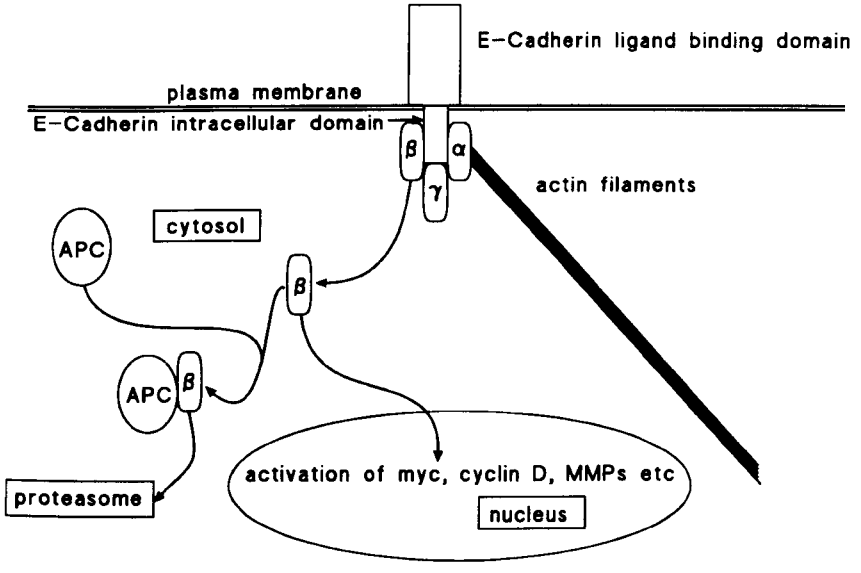


Figure 1.2 Interaction of E-cadherin, β-catenin and APC

1.6.4 Cell cycle control in colorectal cancer

All cells that are continuing to divide pass through the cell cycle. This is closely regulated at a number of stages, to ensure that the number of cells in a population remains stable, and that each daughter cell has one perfect copy of the DNA in the parent cell. One method by which cancer cells escape from normal control is by evading the so-called mitotic checkpoints that regulate the cell cycle. This allows the accumulation of mutations. One study showed that over 90% of colorectal tumours had abnormalities in 2 or more cell cycle control factors (McKay *et al.*, 2002).

Misregulation of growth factors may increase the proportion of cells actively cycling by driving cells from the quiescent state G0 where they are viable, but not dividing, into cycle and then past the 'restriction point' beyond which further mitogenic signalling is not required. The normal cell cycle with the position of the two major checkpoints is illustrated in figure 1.3.

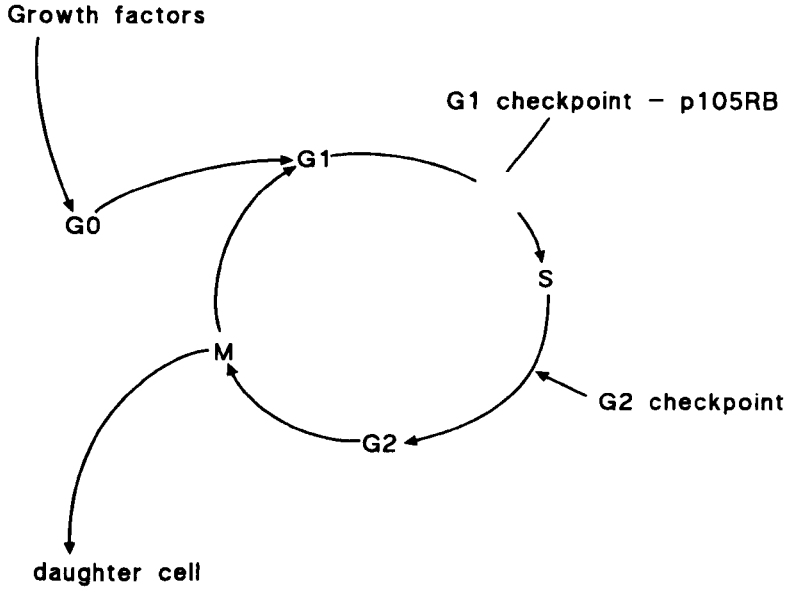


Figure 1.3 The cell cycle. G0 quiescent cells, G1 actively cycling cells, S synthesis of DNA, G2 pause before mitosis, M mitosis, p105RB retinoblastoma protein.

Early in G1, the retinoblastoma protein, p105RB is bound to the transcription factor E2F. During G1, p105RB is phosphorylated by cyclin dependent kinases. This releases E2F, which is then able to cause transcription of genes such as *DNA polymerase,* producing enzymes necessary for DNA synthesis in S phase (Sherr, 1996). Phosphorylation of p105RB is normally prevented by kinase inhibitors such as p16INK. p16INK is commonly mutated in cancer cells, allowing phosphorylation of p105RB, and this releases the cells from the G1 mitotic checkpoint. The same result is also achieved in those tumours with loss of p105RB function.

Cyclin dependent kinase/cyclin complexes are also inhibited by $p21^{WAF1}$. One of the ways in which p53 inhibits cell cycling in the presence of damaged DNA is by acting as a transcription factor, increasing the levels of $p21^{WAF1}$ and thus limiting progress through the cell cycle. Cancer cells without functioning p53 lose this regulatory mechanism (Wang *et al.*, 1999).

The G2 checkpoint controls entry to mitosis. Again, this checkpoint involves the combination of a cyclin dependent kinase (cdc2) and a cyclin (cyclin B), which binds to cdc2, priming it for activation. Final activation depends on the balance between a kinase (wee1) and a phosphatase (cdc25). The breakdown of wee1 at the entry to mitosis allows activation of the complex, which migrates to the nucleus and causes nuclear membrane breakdown. Cyclin genes are frequently over expressed in cancers, pushing the balance towards progress through the cell cycle.

Further checkpoints occur during mitosis (M phase checkpoints) preventing progression of mitosis unless all chromosomes are correctly replicated and aligned on the mitotic spindle. Failure of this mechanism results in aneuploidy, commonly seen in those colorectal cancers developing *via* the microsatellite stable (chromosomal instability) pathway.

1.7 Hypothesis

We have seen that local recurrence is a significant problem for patients with colorectal cancer. It has been well documented that the best predictor of local recurrence is an advanced stage of the primary tumour, and in particular the presence of free malignant cells on the serosal surface of colonic tumours, or circumferential margin involvement in the case of rectal cancers. A considerable amount can be done to improve on the standard of current staging by careful attention to these aspects by the pathologist. However, despite careful pathological examination of the resected specimen, it can sometimes be difficult to be certain whether the serosal surface has been breached. Even careful examination of the specimen cannot give information about what is happening in the peritoneal cavity in the days following surgery, after the specimen has been removed, when tumour cells could be shed from cut lymphatic channels.

We hypothesise that the detection of free malignant cells in the peritoneal cavity during the resection of a colorectal tumour, or in the post-operative drain fluid, could act as a marker for serosal involvement, circumferential margin involvement or the leaking of tumour cells from cut lymphatics into the tumour bed. The presence of such cells would therefore put patients into a group at increased risk of recurrent disease, particularly local recurrence. Given the paucity of work using molecular biological techniques to examine the peritoneal cavity in colorectal cancer, we also hypothesise that the use of such techniques may allow a greater proportion of such patients to be detected.

1.8 Aims

Our primary aim was to describe techniques to permit identification of isolated malignant cells in the peritoneal cavity of patients undergoing surgery for colorectal cancer, both during surgery (in peritoneal washings) and in the first two days after surgery (in post-operative drain fluids).

Secondary considerations were to relate our results to the stage of tumour at presentation, and to follow the patients with both positive and negative results, to attempt to relate our

results to later patterns of recurrence and survival. We wished, if possible, to attempt to quantify any malignant cells detected. Finally, if techniques for detecting cells in this environment were successfully developed, we wished to streamline them as far as possible, to increase the applicability of our results in routine practice.

1.9 Experimental Strategy

In order to achieve these aims we obtained ethical approval to collect samples from patients undergoing surgery for gastrointestinal cancer. Initially we collected samples from those with gastric or oesophageal tumours, as well as colorectal cancer patients. However, the results from the upper gastrointestinal samples were disappointing, perhaps because they have a different mutation spectrum from colorectal tumours. This policy was therefore abandoned, and only samples from colorectal cancer patients were collected.

We decided to attempt to increase the proportion of epithelial cells in our peritoneal samples by subjecting them to an enrichment step using MACS (magnetic activated cell separation). This technique had been used in our laboratory previously for a number of different types of sample from colorectal cancer patients, with considerable success (Wong *et al.*, 1997). The necessity for this step was later considered in a series of experiments recorded in chapter 7.

As described above we elected to use molecular biology techniques to look for malignant cells in our peritoneal samples. This decision was made because of the reported sensitivity of theses techniques in the detection of very small numbers of malignant cells against a background of normal cells. It was also interesting to apply these techniques to a site that had received relatively little attention in colorectal cancer. We elected to use techniques to detect specific DNA mutations, rather than to use RT-PCR based methods. This decision was made because of the disadvantages of using RNA based techniques, described in section 1.5.4.2. We feared these would present particular problems in our situation for several reasons. Firstly, the samples, particularly the drain fluids, were not kept in ideal conditions for the preservation of good quality RNA. Indeed, we did attempt

to extract RNA from some of our samples in order to try to achieve our aim of quantifying any malignant cells detected, and sufficient good quality RNA could not be obtained. Secondly, non-malignant epithelial cells may be present in the peritoneal cavity around the time of surgery, from skin or divided bowel ends, and these would be detected by tests designed to distinguish epithelial cells rather than those with specific mutations. Finally, the problems of illegitimate mRNA expression, leading to false positive results, may be increased by inflammatory stimuli, such as surgery.

Given the paucity of previous data on the use of molecular techniques to detect cancer cells in the peritoneal cavity, it was not possible to make any sensible prediction about the likely rate of positive samples. Because of this uncertainty, we were unable to make a power calculation regarding the number of tumours and peritoneal samples to be studied. We chose to collect as many samples as could be processed by a single worker in the time available.

Among the many different types of test described to detect mutant DNA, we chose to use mainly the Mismatch Ligation Assay (MLA). The reasons for choosing this were primarily practical; papers were available describing clearly the necessary oligonucleotides and reaction conditions to detect common *K-ras* and *p53* mutations (Dong *et al.*, 2001; Jen *et al.*, 1994*b*), which we had selected as our initial targets, and the techniques need to perform the MLA were in use by other workers in our laboratory. The techniques had also been shown to be useful in detecting mutant DNA in a number of different environments (reviewed in section 1.5.4.3.2).

We elected to use the same technique to first look for mutations in the primary tumour samples and then, when a mutation had been detected, to study the peritoneal samples from that patient. This had the advantage of allowing us to develop our mutation detection techniques on samples with adequate amounts and concentrations of DNA, and where we would expect a significant proportion of cells to contain the mutation being studied. One alternative to this strategy would have been to perform direct sequencing on the tumour samples, looking for mutations commonly associated with colorectal cancer,

and then to examine the peritoneal samples for the same mutation with a different technique.

We chose *K-ras* as our initial target mutation because, although it is only mutated in 40% to 50% of tumours, most of these mutations are concentrated at a particular site (codon 12B). About 75% of colorectal tumours have mutations of *TP53,* but these mutations occur at a large number of different sites within the gene. The particular *TP53* mutation we chose to investigate was selected for the practical reason that a cell line with that mutation was available as a positive control.

Rather than continue to repeat the work of others by attempting to detect further *TP53* mutations as described by Dong, we decided to explore other mutations, and the *APC* gene, mutated in almost all microsatellite stable tumours, was an obvious target. Although this a very large gene, with a huge variety of documented mutations, there are two 'hot-spots' (Fearnhead *et al.*, 2001) which are sensible targets for research of this type. After initial difficulties in attempting to design MLA oligonucleotides, described in section 4.7.3, it finally proved possible to detect one of these mutations with a mutant-allele specific PCR.

BRAF was recently shown to be mutated in several forms of human cancer, including about 10% of colorectal cancers. The fact that most of these tumours have a single point mutation (T→A at codon 599) made this an appropriate target for detection with MLA. In addition, this mutation has not been found in tumours with mutations of *K-ras* (this because both activate the same growth factor signalling pathway), and thus the ability to detect *BRAF* mutations would increase the proportion of cases in which we were able to detect a mutation. The design of a MLA to detect *BRAF* mutations is described in section 4.7.4.

CHAPTER 2: MATERIALS AND METHODS

2.1 Patient selection and recruitment

We obtained ethical approval from the South Warwickshire Local Research Ethics Committee (reference number RE464, January 2001). Patients were approached if they were undergoing elective surgery for colorectal cancer, or occasionally, in the early stages of the project, for upper gastrointestinal tumours. A number of patients with benign disease were invited to take part as controls. During the early part of the project we only approached patients if we anticipated that a drain would be placed at operation, but later in the study a number of patients not having drains were included. The consultant surgeons vary in their use of drains for different procedures, but no patient had a drain placed solely because of this project. We explained the reasons for the project, the nature of samples to be collected and the need for review of the notes in the future for follow up. We gave patients an information sheet, which they were encouraged to read and retain. If they agreed to participate in the study, we asked them to sign a consent form. Copies of the patient information leaflet and consent form are given in the appendix, together with the Ethics Committee approval letter.

2.2 Sample collection

Peritoneal washings were collected at the start and end of surgery. Approximately 150-200ml of 0.9% saline (at 37°C when available) was introduced into the peritoneal cavity by the operating surgeon. This was gently agitated for a few seconds and then as much as possible (typically about 100ml) was aspirated with a 60ml catheter tip syringe and collected in a gallipot. After this was handed out from the sterile field we added approximately 800 units of unfractionated sodium heparin. The sample was divided into up to four 50ml sterile Falcon tubes (Sarstedt, Numbrecht, Germany), each containing approximately 25ml of RPMI 1640 tissue culture medium (Gibco BRL, Paisley, UK) with 10% (v/v) foetal calf serum (Life Technologies Ltd., Paisley, UK) and mixed. The addition of the medium and the serum was to provide better transport medium than the saline, which contains only sodium and chloride ions and is unbuffered. The sample was held at 4°C until returned to the laboratory.

The washing at the start of surgery ('pre-operative wash') was obtained as soon as the surgeon had access to the tumour to be resected. On most occasions this was

immediately after opening the abdomen and performing the initial laparotomy, however at times this was delayed by the need to take down adhesions or to perform some preliminary dissection to allow the wash to be instilled around the area of the tumour. For tumours below the peritoneal reflection, the initial wash was made in the pelvic cavity after lifting out the small bowel, but before the pelvic dissection was begun. The wash towards the end of surgery ('post-operative wash') was performed at some convenient time after resection of the tumour, and usually after formation of any necessary anastomoses. We instilled saline into the part of the abdomen or pelvis from which the tumour had been resected, and removed it as described above. For oesophageal tumours, which were resected by a two-stage Ivor-Lewis procedure, the 'pre-operative wash' was performed on opening the pleural cavity rather than in the abdomen.

Once the tumour specimen was removed from the patient it was handed out of the sterile field. Outside the operating theatre it was cut open, with care not to impair the later pathological assessment. Any intestinal contents were washed off with tap water and a small piece of visibly and palpably malignant tissue was excised using scissors. We divided this into 2 and put the pieces into separate freezing vials, which had been pre-cooled in liquid nitrogen. The vials were returned to the liquid nitrogen and transported back to the laboratory where they were stored at $-80^{o}C$ until needed.

At the end of the procedure, if a drain was used, we put a label on the collection bag asking the nursing staff on the ward not to empty the drain, to allow fluid to build up for collection the following morning. Approximately 500 units of the sodium heparin solution were introduced through the drainage port of the collection bag to help to prevent the sample from clotting.

We collected samples from the drain bag or bottle on the first and second post-operative mornings, while the patient was on the ward or intensive care unit. Up to 100ml of drain fluid was collected and any residual fluid was discarded to allow fresh drain fluid to collect over the subsequent 24 hours. Samples were not routinely kept cool while in the drain bag or after collection but were returned promptly to the laboratory for processing.

Information about patient demographics, site of tumour, operation and surgeon's assessment of whether the procedure was curative, was collected at the time of surgery and stored in an Excel spreadsheet. Histological information was collected once the formal report had been prepared and was added to the spreadsheet.

2.3 Treatment of peritoneal washings and drain fluids

We performed density centrifugation and MACS on each sample on the day it was obtained.

2.3.1 Density centrifugation

We removed red blood cells from the peritoneal washings and drain fluids by density centrifugation. Samples in 50ml Falcon tubes were centrifuged at 1600 rpm for 10 minutes. Supernatants were discarded and the cell pellets resuspended in RPMI 1640 medium with 10% FCS. If the sample had been collected in multiple tubes these were combined at this point, and the total volume made up to 15ml. The cell suspension was layered onto 10ml of Lymphoprep (Sodium Diatrizoate 9.1% w/v and Polysaccharide 5.7% w/v, Axis-Shield, Oslo, Norway) in a 25ml universal container (Scientific Laboratory Supplies, Nottingham, UK). This was centrifuged at 1600 rpm for 30 minutes. The mononuclear cell layer at the interface, containing lymphocytes and any epithelial cells present was then aspirated using a Pasteur pipette, taking care not to aspirate Lymphoprep. The aspirated layer was placed in a 15ml Falcon tube and the volume made up to 15ml with RPMI 1640 with 10% FCS. This was centrifuged at 1600 rpm for 15 minutes. The supernatant was discarded, and the pellet resuspended in ice cold PBS. We counted the number of cells retrieved using a haemocytometer (Neubauer improved, Marienfeld, Germany), having first mixed the cells with an equal volume of trypan blue (Sigma-Aldrich, Irvine, UK) to ensure only viable cells were being counted.

2.3.2 Magnetic activated cell separation (MACS)

2.3.2.1 Principle of technique

MACS was developed in Germany in 1990 as a technique to allow separation of cells in suspension into different types, depending on their cell surface markers (Miltenyi *et al.*, 1990). The technique involves the use of magnetic microbeads, which are less than 150nm in diameter. This tiny size means that cell viability and function is not

affected when the microbeads bind to the cell, and cells can be used for cell culture, flow cytometry and other molecular biological techniques after MACS separation.

Cells labelled with microbeads are passed through a magnetisable matrix (in the form of a column), placed in a strong magnetic field, and are trapped in the column. When the column is removed from the magnet the labelled cells are eluted. MACS can be used in two main ways, for enrichment or depletion of cell populations. In enrichment procedures (also called positive selection), microbeads are attached to the cell type of interest, and these cells are initially retained in the magnetised column while unwanted cells run through. The required cells are then eluted after removing the column from the magnet. Positive selection is demonstrated in Figure 2.1a and b. In depletion procedures the 'unwanted' cell type in a mixture is magnetically labelled, and the cells of interest are allowed to run through the magnetised column, with the labelled cells retained.

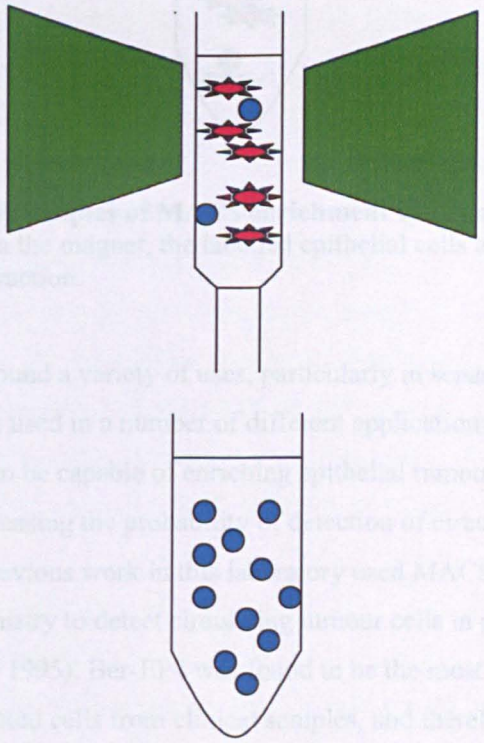


Figure 2.1a Principles of MACS enrichment. Labelled cells (red) are trapped in the magnetised column (magnet shown in green) while unlabelled cells (blue) pass through and are collected as the negative fraction

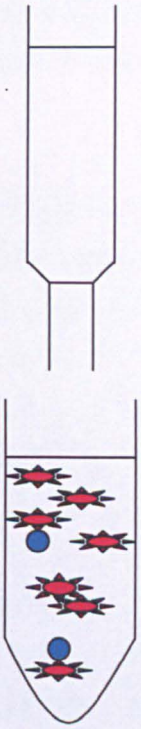


Figure 2.1b Principles of MACS enrichment continued. When the column is removed from the magnet, the labelled epithelial cells are eluted, and collected as the positive fraction.

MACS has found a variety of uses, particularly in separation of lymphocyte subsets. It has also been used in a number of different applications in cancer research, and has been shown to be capable of enriching epithelial tumour cells 10,000-fold in blood samples, increasing the probability of detection of circulating tumour cells (Martin *et al.*, 1998). Previous work in this laboratory used MACS, combined with immunochemistry to detect circulating tumour cells in patients with colorectal cancer (Wong *et al.*, 1995). Ber-EP4 was found to be the most suitable primary antibody for labelling unfixed cells from clinical samples, and therefore was chosen for use in this study. It has also been demonstrated (Latza *et al.*, 1990; Sheibani *et al.*, 1991) that Ber-EP4 does not cross react with mesothelial cells. This is particularly important in

trying to improve the sensitivity of any separation involving samples from the peritoneal cavity, which is lined with mesothelial cells. The disadvantage of using Ber-EP4 is that this antibody is not available directly conjugated to magnetic microbeads. It is therefore necessary to use a two-stage antibody labelling process, using a mouse anti-human Ber-EP4 antibody and then a secondary anti-mouse antibody (usually goat), which is bound to magnetic microbeads.

2.3.2.2 MACS Methods

For most of the peritoneal samples, (washings and drain fluids) the whole sample was subjected to MACS separation. Samples from the last 17 patients were split at this stage, with half being used for MACS and the remainder being left without separation (see chapter 7, Is MACS Necessary?). The protocol for MACS is given in the appendix. Briefly, the cells were washed in ice cold PBS, incubated with the primary antibody (mouse anti-human Ber-EP4, DAKO A/S Glostrup, Denmark), washed again and incubated with the secondary antibody (goat anti-mouse IgG, conjugated to magnetic microbeads, Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). After being washed again, they were resuspended in ice cold MACS buffer (PBS with 2mM EDTA (Gibco BRL) and 0.5% bovine serum albumin (Sigma Aldrich)). Samples were run into a miniMACS column (Miltenyi Biotec) held in a specially designed magnet, and the negative fraction of unlabelled cells was washed through. After removing the column from the magnet, the positive fraction of cells was eluted. For reasons of economy, we reused each miniMACS column once, for samples from the same patient, after washing it with approximately 10 column volumes of ice-cold MACS buffer. We counted the number of cells in both fractions for the first 85 samples, and in the positive fraction only in later samples. Cells in both positive and negative fractions were pelleted by centrifugation at 3000rpm for 8 minutes, the supernatant was removed and the cells stored at –80°C until needed.

2.4 DNA Extraction

2.4.1 Tumour samples

Tumour samples were removed from the freezer and kept on dry ice. We ground each tumour sample, under liquid nitrogen, with a separate pestle and mortar, which had previously been heated to 200°C overnight to destroy any contaminating DNA. The ground tumour was placed in a pre-cooled Eppendorf, and DNA was extracted using

the 'Tissue Protocol' of a Qiagen DNA mini kit (Qiagen, Hilden, Germany). The average lysis time was 3 hours at 56°C. DNA was eluted in buffer AE (as supplied with kit, total volume 400µl) and stored at –20°C until needed. DNA in a number of samples was quantified using a spectrophotometer.

2.4.2 Cell pellets from peritoneal samples

DNA from these samples was extracted using the 'Blood and body fluids spin protocol' of a Qiagen DNA mini kit. Where fewer than 10^5 cells were in any fraction, then 1µl of a solution of polydeoxyadenylic acid (polydA), 5µg/µl (Sigma-Aldrich) was added at the start of DNA extraction. DNA was eluted in buffer AE (between 50 and 200µl, depending on starting number of cells) and stored at –20°C until needed. DNA in a number of samples was quantified using a spectrophotometer.

2.5 PCR (polymerase chain reaction)

We performed PCR to amplify exon 1 of *K-ras*, exon 8 of *p53* and exon 15 of *BRAF*. Details of primer sequences and reaction conditions are given in the appendix. Details about optimisation of PCR reactions and pictures of typical gels are shown in chapter 4. Each PCR performed included a negative control, with no DNA, to detect any contamination. PCR products were mixed with loading dye (orange G (Sigma Aldrich) in 30% glycerol), and loaded into a 1% agarose gel, containing ethidium bromide (Sigma Aldrich, 0.3µg/ml of gel). The products were separated using a voltage of 100 to 150V, depending on size of gel. The gel was imaged under ultraviolet light, and results recorded photographically. If a clear band of the appropriate size was seen after electrophoresis, then the PCR products were frozen at –20°C until needed.

2.6 Mutant allele specific PCR

Mutant- allele specific PCR was used to detect mutations at codon 1309 of the *APC* gene. Details of primers and reaction conditions are given in the appendix; information on optimisation is in chapter 4. Each reaction included a negative control, with no DNA, a positive control, with DNA from a tumour cell line known to have the specific mutation under study, and DNA from a healthy volunteer, as a control for the specificity of the reaction. Each tumour DNA was examined at least twice. The more stringent conditions described in chapter 4 were used to examine tumour DNAs.

The more sensitive but less stringent conditions were used to look for mutant DNA in the peritoneal samples, where DNA concentrations were typically lower.

2.7 Mismatch Ligation Assay (MLA)

2.7.1 Principle of Technique

The Mismatch Ligation Assay (MLA) is a technique based on the ability of two oligonucleotides to anneal adjacent to one another on a complementary DNA strand. These oligonucleotides will only be joined covalently by a DNA ligase if the nucleotides on either side of the join are correctly base-paired. (Landegren et al 1988) This specificity allows single nucleotide mutations to be distinguished.

The relevant segment of target DNA, derived from a primary tumour sample or from a cell pellet from a peritoneal sample is amplified using PCR. The target DNA is denatured, and two oligonucleotides are permitted to hybridise to it. One oligonucleotide is complementary to the mutant DNA sequence being sought (i.e. mutation specific). Up to three possible mutation-specific oligonucleotides can be used in each reaction, being complementary to the three possible non-wild-type bases at the site of mutation. The other oligonucleotide is designed to anneal immediately 3' of the mutation site i.e. with its 5' end adjacent to the 3' end of the mutation-specific oligonucleotide. The 5' end of this oligonucleotide is radiolabelled with ^{32}P. When mutant DNA is present in the target sample the two oligonucleotides can be joined by DNA ligase and the ^{32}P is incorporated into the DNA chain. A blocking oligonucleotide, complementary to the wild type sequence, is present in excess, to prevent any binding of the mutant-specific oligonucleotides to wild type DNA. Alkaline phosphatase is added to the reaction and is able to remove ^{32}P from any oligonucleotides that are not incorporated by the ligase reaction. The reaction products are separated by denaturing polyacrylamide gel electrophoresis and detected using a phosphoimager. Figures 2.2a, b and c illustrate the situation occurring in the presence of mutant DNA, wild type DNA binding to blocking oligonucleotide, and wild type DNA binding to mutation specific oligonucleotide respectively.

Figure 2.2a MLA in the presence of mutant DNA. This shows annealing of mutation specific (shown in red) and end labelled (shown in blue) oligonucleotides to mutant *K-ras* (shown in black, with mutation site in red). The oligonucleotides are joined by T4 ligase, because they are correctly matched, and ^{32}P is incorporated into the DNA chain.

Figure 2.2b MLA with wild type DNA binding to blocking oligonucleotide. This shows annealing of blocking oligonucleotide (shown in green, present in excess) to wild type *K-ras*

Figure 2.2c MLA when mutation specific oligonucleotide anneals to wild type DNA. If a mutation specific oligonucleotide anneals to wild type *K-ras* it cannot be ligated to the end-labelled oligonucleotide because of the mismatch. The ^{32}P is therefore not incorporated and is removed by alkaline phosphatase.

2.7.2 Method for MLA

A detailed protocol for each variation of this assay is given in the appendix, together with details of the suppliers of enzymes. In summary, the relevant oligonucleotide was first labelled with ^{32}P at its 5' end, by incubation with T4 polynucleotide kinase, $^{32}P\gamma ATP$ and the relevant buffer. The T4 kinase was then heat inactivated. We prepared a master mix including mutation specific and blocking oligonucleotides, together with spermidine (Sigma-Aldrich), T4 gene 32 protein (to stabilise single stranded DNA, Roche, Mannheim, Germany) and T4 ligase buffer made to the appropriate volume with 0.9% saline. The relevant PCR product was added to each tube and the labelled oligonucleotide was also added. Each reaction included one sample with PCR product of the relevant exon from a cell line known to have the mutation being studied, as positive control, and one sample with PCR product of the relevant exon from the DNA of a healthy volunteer, as a negative control. The mixture was heated to 95°C for 5 minutes to denature the PCR products and then allowed to cool for 15 minutes at room temperature while annealing occurred. We then added T4 ligase and incubated for 1 hour at 37°C followed by heat inactivation. Alkaline phosphatase with the appropriate dephosphorylation buffer was then added and incubated for 30 minutes at 37°C. Each sample was mixed with a denaturing loading dye, containing formamide, and heated again to 90°C for 5 minutes before being loaded into a pre-warmed 12% denaturing polyacrylamide gel.

Electrophoresis was performed at 300V, with 0.5 x TBE as running buffer. The samples were run as far as possible, to achieve maximum separation between reaction products and the solvent front. After electrophoresis we soaked the gel in 10% acetic acid, 20% methanol for 10 minutes and then 20% glycerol for 30 minutes. The gel was dried overnight under 25mmHg vacuum with heating to 80°C for the first 3 hours of drying. Gels were wrapped in cling film and placed in a previously 'blanked' phosphoimager cassette for approximately 4 hours. Images were collected using a Fuji phosphoimager and analysed using Image Quant software.

2.8 Cell culture

The cell lines shown in table 2.1 were maintained in culture to provide material for spiking experiments and DNA for use as positive controls. All these cell lines are

derived from human colorectal tumours. All cells were grown in the medium shown, with the addition of 10% v/v foetal calf serum (Life Technologies Limited), 2% v/v 200mM L-glutamine (Gibco BRL) and 500U penicillin and 500µg streptomycin (Sigma-Aldrich). RPMI 1640 medium (without L-glutamine, with Na HCO_3) was obtained from Gibco BRL, and L-15 medium, without L-glutamine was obtained from Sigma-Aldrich.

All cell lines were maintained in an incubator at 37°C with 5% carbon dioxide. The medium was changed once or twice per week and the cells were subcultured when confluent, after trypsinisation. Cell lines grown in L15 medium were cultured in non-vented flasks; all others were cultured in vented flasks.

Cell line	ATCC No.	Source	Medium used	Mutation
HT-29	HTB-38	Dr A Morris	RPMI 1640	P53 codon 273, BRAF codon 599
SW480	CCL-228	ATCC	L-15 and RPMI 1640	P53 codon 273, K-ras codon 12
LS1034	CRL-2158	ATCC	RPMI 1640	APC codon 1309
COLO 205	CCL-222	Dr A Morris	RPMI 1640	BRAF codon 599
SW1417	CCL-238	Dr A Morris	L-15	APC codon 1450, BRAF codon 599

Table 2.1 Table showing details of cells maintained in culture.

Information about particular mutations found in various colorectal cancer cell lines was found on the website of the American Type Culture Collection (ATCC, www.atcc.org) or from the literature (Fearnhead *et al.*, 2001; Gayet *et al.*, 2001)

CHAPTER 3: PATIENT DEMOGRAPHICS AND HISTOLOGY

3.1 Patient Selection and Patient Details

Samples were collected from 58 patients undergoing major abdominal surgery at Warwick Hospital between 25/4/01 and 11/9/02. Operations were performed by one of the 3 surgeons having a special interest in gastrointestinal surgery and only patients having elective procedures were included, to allow sufficient time for informed consent to be obtained. Only 2 patients declined to take part in the research project, both were extremely anxious about their forthcoming surgery.

Of the 58 patients, 53 underwent surgery for neoplastic disease. After some early samples were used to prepare techniques, 50 tumour samples from 47 patients were searched for mutations. These were 44 colorectal cancers, 2 colorectal adenomas and 4 upper gastrointestinal cancers, 3 oesophageal and one gastric. As discussed in section 1.9 the patients with upper gastrointestinal tumours are not considered further, because their tumours were not amenable to analysis using the same tests applied to the colorectal tumours. Samples from the remaining 5 patients, who underwent surgery for benign diseases, were used to optimise techniques and to assess sensitivity after adding cells from cultured colorectal cancer cell lines. Details of the 43 patients with tumours used in the study are shown in table 3.1, together with information about the type of tumour (adenoma or cancer) and the operation performed.

Details of the patients with benign diseases, whose samples were used as controls, are shown in table 3.2.

tumour no	age	sex	op date	diagnosis	operation performed
1	73	female	08/05/2001	CRC	right hemicolectomy
2	82	female	12/06/2001	CRC	right hemicolectomy
3	67	male	14/06/2001	CRC	anterior resection
4	66	female	19/06/2001	Adenoma	anterior resection
5,6*	60	male	19/06/2001	CRC/CRC	panproctocolectomy
8	84	male	28/06/2001	CRC	sigmoid colectomy
9	71	female	05/07/2001	CRC	anterior resection
10	75	male	18/07/2001	CRC	anterior resection
12	78	male	06/09/2001	CRC	panproctocolectomy
13	74	female	11/09/2001	CRC	completion colectomy
14	74	male	26/09/2001	CRC	anterior resection
16	62	male	05/10/2001	CRC	abdominoperineal resection
17	56	male	11/10/2001	CRC	anterior resection
18	77	female	18/10/2001	CRC	anterior resection
19	81	female	04/12/2001	CRC	right hemicolectomy
20	78	male	05/12/2001	CRC	anterior resection
21	73	male	08/01/2002	CRC	extended right hemicolectomy
22	57	female	12/03/2002	CRC	right hemicolectomy
23	74	male	13/03/2002	CRC	SB resection & Hartmanns
24	70	female	19/03/2002	CRC	left hemicolectomy
25	80	male	19/03/2002	CRC	sigmoid colectomy, SB resection
26	59	female	25/03/2002	CRC	anterior resection & hysterectomy
27	63	female	28/03/2002	CRC	anterior resection
28	75	female	08/04/2002	CRC	right hemicolectomy
29	67	male	10/04/2002	CRC	anterior resection
30	82	male	11/04/2002	CRC	abdominoperineal resection
31	68	female	16/05/2002	CRC	anterior resection & hysterectomy
32	73	female	19/06/2002	CRC	anterior resection & hysterectomy
33	54	male	26/06/2002	CRC	extended right hemicolectomy
34	73	male	26/06/2002	CRC	right hemicolectomy
35	60	female	27/06/2002	CRC	left hemicolectomy
36,37*	55	male	10/07/2002	CRC/CRC	left hemicolectomy
38,39*	73	male	25/07/2002	Adenoma/CRC	right hemi. and anterior resection
40	56	male	30/07/2002	CRC	left hemicolectomy
41	54	female	31/07/2002	CRC	right hemicolectomy
42	52	male	01/08/2002	CRC	anterior resection
43	54	female	06/08/2002	CRC	left hemicolectomy
44	75	male	08/08/2002	CRC	left hemi. & SB resection
45	77	female	21/08/2002	CRC	anterior resection
46	68	female	04/09/2002	CRC	abdominoperineal resection
47	84	male	09/09/2002	CRC	anterior resection
48	76	female	10/09/2002	CRC	extended right hemicolectomy
49	73	male	11/09/2002	CRC	right hemicolectomy

Table 3.1 Information about the patients with colorectal tumours recruited to the study. CRC colorectal cancer, SB small bowel, * 2 tumours from same patient

control no.	age	sex	operation date	diagnosis	operation
1	52	male	03/05/2001	colitis	panproctocolectomy
2	60	female	12/06/2001	crohns disease	right hemicolectomy
3	53	female	05/07/2001	colitis	proctectomy
4	37	male	12/07/2001	diverticular disease	sigmoid colectomy
5	76	female	05/09/2001	diverticular disease	anterior resection

Table 3.2 Information about participating patients with benign diseases.

3.2 Pathological Data

Data were collected in a standardised way from the pathology reports issued by the histopathologists at Warwick Hospital. Although most specimens were reported on by a pathologist with a special interest in gastrointestinal tumours this was not universal. In the later part of the study, colorectal cancer specimens were reported according to a standard minimum data set. Information was collected on degree of tumour differentiation, presence of a mucinous component, modified Dukes' stage, TNM stage, macroscopic invasion of adjacent organs, whether preoperative treatment had been given with chemotherapy and/or radiotherapy and whether the surgeon considered that the operation was curative, palliative or uncertain. This data collection was modified for adenomas. Table 3.3 shows the pathological data concerning the cancers collected, and table 3.4 shows data about the adenomas.

Tumour number	Dukes' stage	Differentiation	TNM stage	surgeon's assessment	comments
1	A	mucinous	T3N0MX	curative	very high CEA pre operatively
2	B	moderate	T3N0MX	uncertain	clinically T4, stuck to ileum
3	D	moderate	T3N2M1	palliative	pre-operative radiotherapy
5	B	well/moderate	T3NOMX	uncertain	
6	B	well/moderate	T3NOMX	uncertain	
8	B	moderate	T3N0MX	uncertain	
9	A	moderate	T2N0MX	curative	
10	A	moderate	T1N0MX	curative	
12	D	moderate	T3N1M1	palliative	multiple large polyps
13	B	moderate	T3N0MX	curative	APR 1996. Tumour stuck to SB
14	B	moderate	T3N0MX	curative	
16	C2	poor	T2N2MX	curative	previous local excision (pT2)
17	B	moderate	T3N0MX	curative	
18	C1	moderate	T3N1MX	curative	
19	A	moderate	T2N0MX	curative	
20	C1	moderate	T2N2MX	curative	
21	C1	mucinous/moderate	T3N2MX	curative	
22	C1	well/moderate	T3N1MX	curative	
23		moderate		palliative	recurrent CRC. R hemi. Jan 2001
24	B	moderate	T4N0MX	uncertain	local perforation
25	D	moderate	T4N2M1	palliative	perforation. Bladder & SB stuck
26	B	mucinous/moderate	T4N0MX	palliative	perforation. Invading uterus
27	D	moderate	T4N2M1	palliative	local abscess
28	B	moderate	T3NOMX	curative	
29	B	mucinous/moderate	T3N0MX	curative	
30	C1	moderate	T3N1MX	curative	
31	B	moderate	T4N0MX	uncertain	abscess. Stuck to POD/vagina
32	B	moderate	T3N0MX	curative	pre-op radiotherapy/ defunctioned
33	C1	moderate	T3N1MX	curative	trace of ascites
34	B	moderate	T3N0MX	curative	
35	C1	moderate	T3N1MX	curative	ascites, perineural invasion
36	D	moderate	T3N1M1	palliative	bladder involved
37	D	moderate	T3N1M1	palliative	bladder involved
39	B	moderate	T3N0MX	curative	incidental splenectomy
40	C2	moderate	T3N2MX	curative	
41	B	moderate	T3N0MX	curative	
42	D	moderate	T3N0M1	palliative	
43	C1	moderate	T2N1MX	curative	previous breast cancer
44	D	moderate	T4N1M1	palliative	invading SB through to mucosa
45	C1	moderate	T3N2MX	curative	close circumferential margin
46	A	mucinous/moderate	T2N0MX	curative	pre-operative radiotherapy
47	B	moderate	T3N0MX	curative	
48	C2	mucinous/poor	T4N2MX	curative	separate peritoneal nodule with ca
49	A	moderate	T1N0MX	curative	submucosal vascular invasion

Table 3.3 Pathological data for colorectal cancers collected. APR abdominoperineal excision of rectum, CEA carcinoembryonic antigen, POD pouch of Douglas

tumour no.	type	degree of dysplasia
4	villous adenoma	severe
38	tubulo-villous adenoma	moderate/ severe

Table 3.4 Pathological features of adenomas.

3.3 Are our Study Patients a Representative Sample?

Considering only those patients with primary colorectal cancer there were 42 patients, 23 men and 19 women (male: female ratio 1.2:1) of mean age 69.5 years. A recent large national audit of patients presenting with colorectal cancer in the UK (Tekkis, 2002) included data from over 8000 patients. The median age at presentation in this audit was 72 years (although the audit also included patients treated as emergencies or not undergoing surgery, who tend to be older) and the male: female ratio was 1.24:1. Our patient population is therefore well matched in age and sex distribution to the typical UK population with colorectal cancer.

Table 3.5 compares the distribution of colorectal cancer sites between the tumours in the national audit and the 43 primary colorectal tumours in our study.

Site of tumour	National audit	Tumours in study
Rectum	32%	16/43 (37%)
Sigmoid/left colon	36.5%	14/43 (33%)
Right/transverse colon	31%	13/43 (30%)

Table 3.5 Sites of colorectal tumours in National audit and this research.

The higher proportion of rectal cancer patients in our study could represent a difference in classification of tumours at the rectosigmoid junction. It could also represent a bias in recruiting patients. Surgery for rectal cancer involves pelvic dissection and therefore drains are more commonly inserted. Patients undergoing rectal cancer surgery were therefore more likely to be recruited to the study.

A comparison of Dukes' stage of those patients in the national audit undergoing surgery and the patients in our study is shown in table 3.6.

Dukes' stage	National audit	Patients in study
A	795/6247 (12.7%)	6/43 (14%)
B	2276/6247 (36.4%)	17/43 (40%)
C	2189/6247 (35%)	12/43 (28%)
D	987/6247 (15.8%)	8/43 (19%)

Table 3.6 Analysis of study patients by Dukes' stage compared with national audit

It can be seen that the Warwick patients are well matched in terms of stage with the data collected in the National Audit.

The surgeon rated each patient at the time of operation as to whether the resection was felt to be curative in intention, clearly palliative (multiple liver metastases or obvious residual tumour) or uncertain. 28 patients (67%) had surgery thought to be curative, 21% (9 patients) had a palliative resection, and the surgeon was uncertain in the remaining 5 patients. These figures are similar to a large Scottish audit in which 70% of patients had surgery thought to be curative (McArdle and Hole, 2002).

Both of the adenomas that were resected were severely dysplastic at least in part, and neither was amenable to colonoscopic removal. The caecal adenoma was resected during the same operation as excision of a rectal cancer by anterior resection.

3.4 Samples Collected

Tumour samples, usually in duplicate were collected from all patients with cancer or adenomas. A peritoneal washing at the start of surgery was collected from each patient, and a wash at the end of surgery from all except one patient. One of the samples at the start of surgery consisted of ascites rather than peritoneal wash fluid. In 7 patients an abdominal drain was not used (intentionally in 6, surgeon forgot in 1). 2 patients' drains produced no output on either the first or second post-operative day, and in 2 others nothing drained between 24 and 48 hours after surgery. In 2 patients a special type of abdominal drain connected to the wall suction unit was used. This was found to cause lysis of the cells and therefore drain samples from these 2 patients were not analysable. It

was not always possible to collect drain fluid samples on the second post-operative day if this fell on a weekend.

For the 50 tumours studied (including the upper gastrointestinal tumours), one paired peritoneal sample was available in 1 case, 2 samples in 10 cases, 3 samples in 21 and the complete set of peritoneal washings and first and second day drain fluids in 18. A median of three peritoneal samples was available for comparison with each of the tumour samples collected.

3.5 Follow up data

To reduce demands on participating patients, no specific follow up appointments or investigations were required as part of this study. Follow up data therefore represents the latest information available in the patients' records, after clinical review, or the investigations ordered routinely, or in response to patients symptoms, by the consultant surgeons concerned. Data regarding length of follow up, adjuvant treatment received and whether patients have suffered from recurrent disease are given in table 3.7. The variable length of follow up in patients having their operations at similar times represents differences in follow up regimes. Some data may be in case notes of the regional oncology centre and therefore unavailable for this study. Of the 43 patients, 5 have died. One patient suffered a myocardial infarction (MI) in the immediate post-operative period and died shortly after discharge from hospital. Two of the others had metastatic disease at presentation, one other suffered local recurrence (Dukes' B, pT3 tumour with clinical small bowel involvement) and another, with a Dukes' C tumour suffered from liver metastases. 10 patients are alive with metastases from their colorectal cancer, and one patient has metastatic malignant melanoma. 27 patients (63%) are alive without evidence of recurrent cancer; the median follow up for this group is 10 months, range 3-21 months.

Tumour number	Length of follow up (months)	Adjuvant treatment	Status
1	15		Alive with disease (liver metastases)
2	21		A&W
3	21		Alive with disease (liver, lung)
4	5		A&W
5 & 6	16		A&W
8	4		A&W
9	19		A&W
10	15		A&W
12	14		Died with disease (liver metastases)
13	10		Died with disease (local recurrence)
14	18		A&W
16	18		Alive with disease (inguinal nodes)
17	17		Alive with disease (liver, ?pelvis)
18	17		Alive with disease (liver metastases)
19	10		A&W
20	<1		Died, post-operative MI
21	10	Chemotherapy	A&W
22	7	Chemotherapy	Died with disease (liver metastases)
23	3	Chemotherapy	Alive with disease (peritoneal)
24	10	Chemotherapy	A&W
25	6		Alive with disease (liver metastases)
26	9	Chemotherapy	A&W
27	11		Died with disease (pelvic recurrence)
28	10		A&W
29	10		A&W
30	10		A&W
31	11	Radiotherapy	Alive, no disease but complications of surgery
32	9		Alive, metastases from malignant melanoma
33	4	Chemotherapy	A&W
34	12		A&W
35	12		A&W
36 & 37	6		Alive with disease (liver metastases)
38 & 39	9		A&W
40	3	Chemotherapy	A&W
41	7		A&W
42	10	Chemotherapy	Alive with disease (liver metastases)
43	6	Chemotherapy	A&W
44	6		Alive with disease (liver metastases)
45	6		A&W
46	6		A&W
47	6		A&W
48	7	Chemotherapy	A&W
49	6		A&W

Table 3.7 Follow up data for study patients. A&W= alive and well, with no known recurrent disease.

3.6 Discussion

We have described a group of 43 patients with 46 colorectal tumours, 43 primary cancers, 2 adenomas and 1 recurrent tumour. These patients are of similar age and sex distribution to the UK population with colorectal cancer, and have disease of similar stage. 63% of these patients are alive without evidence of recurrent disease after a median follow up period of 10 months. Evidently a longer follow up is necessary before any conclusions can be drawn concerning the risk of recurrence if tumour cells are detected in the peritoneum.

CHAPTER 4: DEVELOPMENT OF PROTOCOLS AND VALIDATION OF TECHNIQUES

4.1 Introduction

As discussed in section 1.9 (experimental strategy) we chose to use magnetic cell separation to enrich the epithelial cells in our samples, and then molecular biological techniques to search them for DNA with specific mutations known to occur in colorectal cancer. It was important to ensure at each stage of the experiments that the techniques we were using were capable of allowing detection of mutant DNA in the relevant samples.

To ensure that we were able to detect such mutant DNA, when it was present in small amounts against a background of large amounts of normal DNA (a situation we could expect in our clinical samples) we performed a series of 'spiking' experiments. These involved adding known numbers of cells from cancer cell lines to samples from patients undergoing surgery for benign diseases, or to peripheral blood mononuclear cells from healthy volunteers working in the laboratory. Different cell lines were required for the various mutations under study. Details of the cell lines used, and the mutations present in each cell line are given in section 2.8. Spiking experiments were performed at each stage of our protocols to assess the sensitivity of our results.

Another important consideration, especially during the early stages of processing the samples (density centrifugation and MACS analysis) was to ensure that we were not destroying cells by repeated manipulation such as washing and centrifugation. If excessive numbers of cells were lost during these processes then the chances of our detecting mutant DNA would be decreased. We therefore made efforts, particularly with the earlier samples, to count the number of cells present at each stage, to assess them for viability and to calculate the proportion being lost.

4.2 Density Centrifugation

To ensure that a satisfactory proportion of mononuclear cells were recovered after density centrifugation, about 10ml from a number of the early peritoneal washing samples was

sent for cell counting on the coulter counter of the Haematology Laboratory at Warwick Hospital (by kind permission of Dr Peter Rose). Table 4.1 shows the results, with the rate of recovery varying from 20% to 150%.

	CONCENTRATION WBC IN SAMPLE	VOLUME IN ML	TOTAL WBC	WBC AFTER LYMPHOPREP	PERCENTAGE RECOVERED
1	0.1×10^9/l	85	8.5×10^6	1.3×10^7	153%
2	0.1×10^9/l	100	10^7	2×10^6	20%
3	0.2×10^9/l	100	2×10^7	9.5×10^6	48%

Table 4.1 Recovery of mononuclear cells after density centrifugation. WBC = white blood cells.

Why are these results so variable? Firstly, the coulter counter in the clinical laboratory is not set up to allow such small numbers of white cells to be counted routinely. The concentrations in our samples are so low because they consist of bloody fluid, which is then diluted with the 100ml of saline used to retrieve the sample. This low concentration may lead to inaccuracies, with values less than 0.1×10^9/l automatically being rounded up. Secondly, the numbers of lymphocytes in the samples sent to the laboratory were too low to register. It is mononuclear cells that are preferentially concentrated by Lymphoprep. Finally, there was a tendency for the early samples in the series to clot before they were returned to the lab for processing. This was overcome later by the addition of heparin after collection and before storage and transport. If a sample was particularly clotted this could cause an unexpectedly low recovery rate because a significant number of cells would be discarded with the clots.

In the case with more than 100% recovery, this may be due to contamination by erythrocytes. These are typically 1-5% of total cells recovered using Lymphoprep (product datasheet), but at times are clearly visible as red cell contamination of the pellet and may be more than this. Excessive recovery may also represent inaccuracies in counting with the haemocytometer.

These results were sufficient to convince us that the number of cells being recovered was of the same order of magnitude as the number of cells in the sample initially, and given the limitations outlined above further samples were not sent for counting.

4.3 Numbers of Cells in Samples

There was a wide range of numbers of nucleated cells, recovered after density centrifugation, from the various types of sample collected. Overall, the samples contained a median of 1.6×10^7 cells, (n = 157) with a range of 2.2×10^5 cells to 4×10^8 cells (mean 3.2×10^7). If the 10 extreme high and low values are discarded the mean number is 2.5×10^7 cells per sample with a range from 1.5×10^6 to 1×10^8. Even without the extreme values these numbers differ by a factor of 67.

The numbers of cells in the samples varied over the time course of sample collection. The median number of cells (in millions) was 8.5 in pre-operative washes, 17 in post-operative washes, 47 in day 1 drain fluids and 20 in day 2 drain fluids. There were significantly more cells in the day 1 drain fluid than in the pre-operative ($p<0.001$) or post-operative washes ($p<0.01$, Dunn's multiple comparisons test). These figures are as expected from observation of the samples, which tended to be relatively lightly bloodstained at the start of surgery (especially when diathermy was used to open the abdomen), bloodier by the end of surgery and heavily bloodstained on the first post-operative day. The first day drain fluids may also contain proportionally more white blood cells given the major inflammatory stimulus of surgery.

The number of cells in the Ber EP4-positive fraction after MACS also varied widely. The median value for the 160 samples was 1.2×10^5 cells, range 0 to 1.1×10^7 (mean 6.3×10^5). Again, excluding the 10 extreme high and low values gives a mean number of 3.7×10^5, with a range of 2×10^4 to 2.3×10^6.

The proportion of cells in the positive fraction after MACS is important in calculating the enrichment obtained by the process, and in determining whether MACS is helpful or necessary. The median value for the proportion of cells in the positive fraction (n = 157)

was 1.03% with a range of 0 to 33% (mean 2.5%). Excluding the extreme values as before the mean is 1.5% and the range 0.18% to 8.3%. Thus for a sample with the median proportion of cells in the positive fraction, it appears that an enrichment of almost 100 fold occurs after MACS separation. This enrichment is only achieved if we assume that all the epithelial cells are present in the positive fraction after separation. Although work in our laboratory has demonstrated that excellent separation of epithelial cells from other cell types can be made using MACS techniques (V Swales, personal communication, separation of epithelial cells from disaggregated tumour samples), it is likely that the recovery of epithelial cells from these samples is less complete, simply because there are so few of them.

4.4 Recovery of Cells with MACS

The MACS process described (see section 2.3.2 and appendix) involves four centrifugation steps and two incubations. The cells are also passed through a column and then forcibly washed out. Each of these steps could cause cells to be damaged or lost, and it was important for us to clarify whether or not significant numbers of cells were affected. For the first 85 samples we therefore performed counts of the number of cells retrieved after density centrifugation and the numbers in the positive and negative fractions after MACS. The first count was compared with the sum of the other two, to give the percentage of cells recovered. The median recovery after MACS was 70%, range 5% to 208%, interquartile range 44% to 100%.

For samples with up to 5×10^7 cells (87% of samples) before MACS, there was a correlation between the number of cells in the initial sample and the recovery rate, with a greater proportion of cells recovered from larger samples ($r = 0.3491$, $p = 0.0023$). This correlation is shown in figure 4.1.

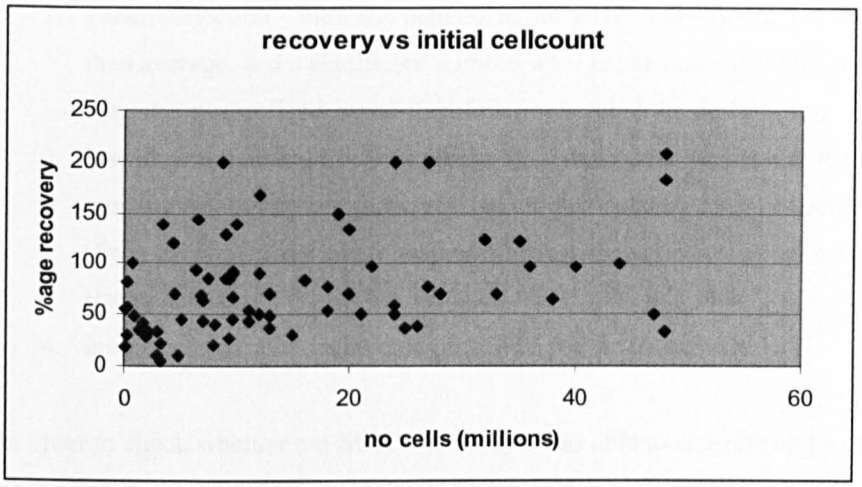


Figure 4.1 Graph showing percentage recovery of cells after MACS against number of cells present in sample after density centrifugation (for samples with up to 5 x 10^7 cells).

If samples larger than 5 x 10^7 cells are included then this correlation is lost. This may be because larger samples exceed the capacity of the miniMACS columns, and a greater proportion of cells are trapped within the column at the end of the process. Very large samples therefore have a lower rate of cell recovery than moderately large samples.

4.5 Is MACS Effective In Enriching Epithelial Cells?

Although MACS has previously been shown to be effective in enriching colorectal cancer cells from blood and from tumour cell suspension, it was important to demonstrate that the particular conditions we had chosen to use were effective in our samples. Cytospin slides were prepared from the positive fractions of 38 samples, and stained with haematoxylin and eosin. These were examined by Dr Mark Newbold, consultant histopathologist at University Hospital Coventry and Warwickshire. No clearly malignant cells were seen in these samples. Given previous results in our laboratory we would have expected some positive results in this number of samples. The possible reasons for our failure to see cells include –

1. Inadequacy of MACS technique

2. Patient selection – the early patients in our series had more favourable tumours than average, and a significant number were rectal cancers, which may be below the peritoneal reflection and therefore not involve the peritoneum.
3. Insufficient material from the positive fraction being loaded into the cytospin preparation- in an attempt to preserve enough cells for later molecular analysis (from the sometimes small positive fractions) many of the slides contained only scanty cells.
4. Inadequate staining technique – resolved for the later slides.

In order to check whether our MACS technique was able to separate epithelial cells from peritoneal washings and drain fluids we performed spiking experiments using cells from the colorectal cancer cell lines HT29 and SW480, which were maintained in culture in the laboratory. Varying numbers of cancer cells were introduced into samples from patients with benign conditions undergoing surgery of similar magnitude to a cancer resection (see chapter 3). Between 10^5 and 10^2 cells were added to samples (typically 100ml in volume) before density centrifugation, and these were then processed using the same protocols as the patient samples. Cytocentrifuge slides were prepared from all these samples, in some cases using only a little of the available material from the positive MACS fraction, and in other cases using the whole positive fraction either by repeated loading of the cytospin chamber or by preparing several different slides. When not all cells were used to make slides the remainder were pelleted and frozen at –80°C. DNA from these pellets was used to assess the sensitivity of molecular techniques at a later stage (see section 4.8). After fixation and haematoxylin and eosin staining the slides were examined. Cancer cells were identified by their large size and high nuclear to cytoplasmic ratio. Although some of the slides were of poor quality or contained only scanty cells, cells thought to be from the cell lines were seen after 10 cells were added per millilitre of initial sample, suggesting adequate enrichment by the MACS process. Examples of slides obtained from the positive and negative fraction are shown in figures 4.2 and 4.3.

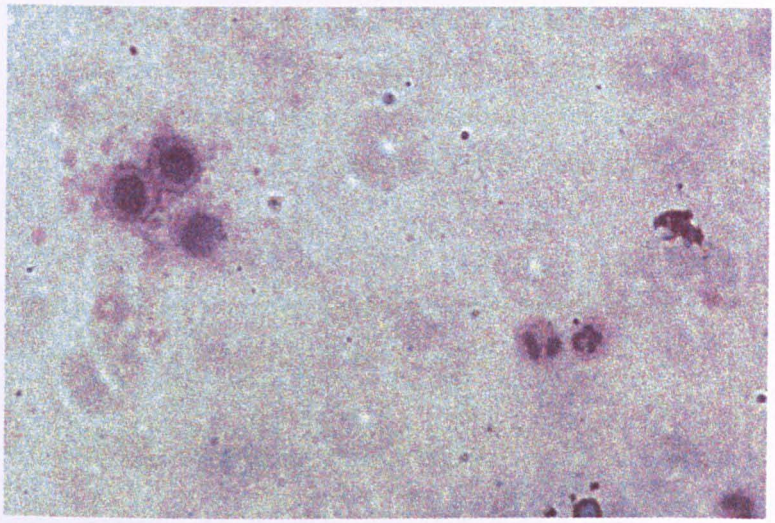


Figure 4.2 Positive fraction after MACS separation. A group of large cells, with large, deeply staining nuclei is seen (haematoxylin and eosin staining).

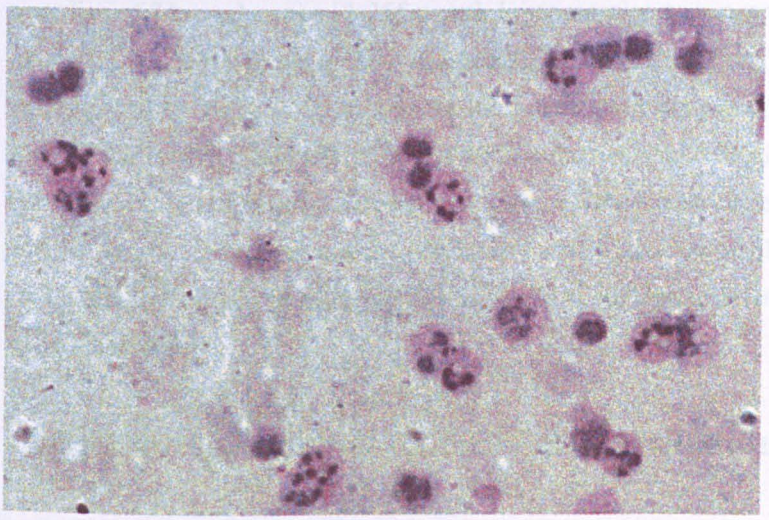


Figure 4.3 Negative fraction after MACS separation. A large number of leucocytes are seen (haematoxylin and eosin staining).

These pictures suggest that the populations of cells in the positive and negative fractions after MACS are different, with the positive fraction containing the malignant cells added to the sample as well as some white blood cells that become trapped in the column. We thus appear to be achieving our aim of enriching the epithelial cell population in our samples, and thus increasing the chance of detecting small numbers of malignant cells in our patient samples.

Further evidence that MACS provides enrichment of cancer cells in peritoneal samples is given in Chapter 7 'Is MACS necessary?'

4.6 DNA Extraction And Quantification

DNA was extracted from tumour samples and from frozen cell pellets derived from peritoneal samples as described in sections 2.4.1 and 2.4.2. The concentration of DNA extracted from the first 19 tumours was quantified using the spectrophotometer, and was a median of 74μg/ml, range 13-265 μg/ml. Later samples were assessed for the presence of amplifiable DNA by using them in an established PCR reaction (for *K-ras*, *TP53* or wild type *APC*). Three of the tumour samples gave DNA that would at times fail to amplify with PCR, in one case despite DNA extraction from a duplicate sample. Two peritoneal samples did not yield amplifiable DNA; in a further three samples either the MACS positive fraction or the non-separated fraction failed to amplify. Considering the small numbers of cells in many of the samples, it is perhaps surprising that so few failed to give usable DNA.

4.7 Polymerase Chain Reaction (PCR) Optimisation and Sequencing

PCR is a technique that is able to amplify a particular segment of DNA millions of times in a matter of hours. Each PCR reaction requires specific concentrations of reagents in order to give optimal amplification but maintain specificity. The optimisation process for each reaction may require variation in magnesium concentration, annealing temperature of number of cycles as well as concentration of enzymes or dNTPs.

Taq polymerase requires free magnesium for activity. The amount of free magnesium depends on other reaction components such as template DNA. If excess magnesium is present then enzyme fidelity is reduced. It is therefore important to use the lowest concentration of magnesium that allows for reliable amplification of DNA. This varies with each reaction, and indeed may vary for the same reaction with different brands of enzyme (see below). Initial difficulties in obtaining reliable amplification were due to a failure to realise that magnesium chloride solutions form a concentration gradient on freezing and require thorough mixing before use.

The temperature used during the annealing phase of each PCR cycle also needs to be adjusted for each reaction. The melting point (T_m) of the primers gives a guide to the likely temperature required, with attempts at optimisation beginning with annealing temperatures about 5°C below the T_m. A high annealing temperature increases the amount of specific product formed and reduces primer-dimer formation.

Once a PCR reaction is producing a product, it is necessary to ensure that the correct portion of DNA is being amplified. The size of band produced after gel electrophoresis can be compared with a standard ladder. The appropriate size can be predicted from knowledge of the sequence of the DNA to be amplified. The PCR products can also be analysed by DNA sequencing. The resulting sequence can be compared with the expected sequence, if known, or can be checked using programmes such as the National Center for Biotechnology Information's BLAST programme (NCBI BLAST).

The optimisation for each PCR reaction performed, and sequencing data to confirm that the appropriate section of DNA was being amplified are presented below. Details of the optimum conditions for each reaction (magnesium chloride concentration and cycle times and temperatures) are given in the appendix.

4.7.1 *K-ras*

Primer sequences were obtained from the literature (Sidransky *et al.*, 1992). A magnesium concentration of 3mM was found to give reliable amplification; results were unpredictable when lower concentrations were used. A band of approximately 200 base pairs was seen after electrophoresis in a 1% agarose gel. The predicted size of the fragment was 202 base pairs. Figure 4.4 shows the result of a typical PCR for *K-ras*.

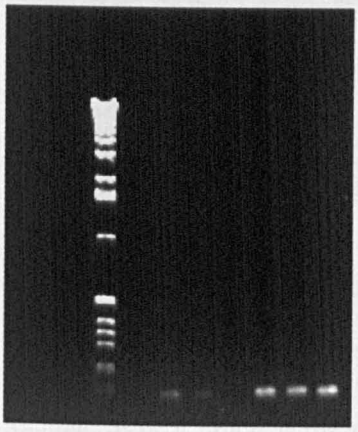


Figure 4.4 Typical gel showing result of PCR to amplify *K-ras*. Lane 1: 1kb ladder, lane 2: negative control, lanes 3-8: DNA from various tumour samples.

Samples of PCR products from this reaction were submitted for sequencing analysis and this confirmed that *K-ras* was being amplified. To detect mutations of codon 12 more clearly on sequencing analysis, alternative primers, obtained from Shoo Yee Wong, giving a larger PCR product (approximately 400bp) were used. Primer sequences and reaction conditions are described in the appendix. An example of a sequencing result for *K-ras* is shown in figure 5.2.

4.7.2 *TP53*

The primer sequences for the amplification of exon 8 of *TP53* were obtained from the literature (Dong *et al.*, 2001), as were the cycle times and temperatures. These are detailed in the appendix. A magnesium concentration of 3mM was found to give good

amplification without primer-dimers. A PCR product of between 150 and 200 base pairs was obtained. A typical result of a PCR to amplify exon 8 of p53 is shown in figure 4.5.

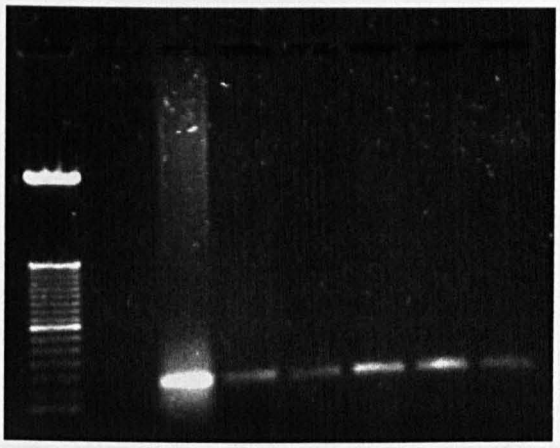


Figure 4.5 Typical gel showing result of PCR to amplify *TP53*. Lane 1: 50bp ladder, lane 2: negative control, lane 3: positive control (DNA from HT29 cell line), lanes 4-8: DNA from various HT29-spiked peritoneal samples

Samples of PCR products from this reaction were submitted for sequencing analysis, and the resulting sequence analysed using the NCBI BLAST programme. The sequence obtained was shown to be that of human *TP53*, confirming the specificity of the primers. An example of a sequencing result from a tumour with a *TP53* mutation is shown in figure 5.5.

4.7.3 *APC (adenomatous polyposis coli* gene)

Initially primers were designed to amplify part of the mutation cluster region of the *APC* gene including the two commonest mutation sites, codons 1309 and 1450. Details of these mutations were obtained from the literature (Fearnhead *et al.*, 2001) and confirmed on a website containing a database of *APC* mutations:
http://p53.curie.fr/p53%20site%20version%202.0/APCdatabase.html#Anchor-47857

The DNA sequence of this exon was obtained from the NCBI website:
http://www.ncbi.nlm.nih.gov/mapview/seq_reg.cgi?chr=5&from=112490538&to=112628882

The relevant portion was submitted to the 'primer designer' programme. A set of primers was selected (see appendix) and, because their T_m was similar to those of the *TP53* primers the same reaction conditions were used and found to be satisfactory. The predicted size of the PCR product was 536 base pairs, and as this figure shows, the band obtained was between 517 and 550 base pairs in length.

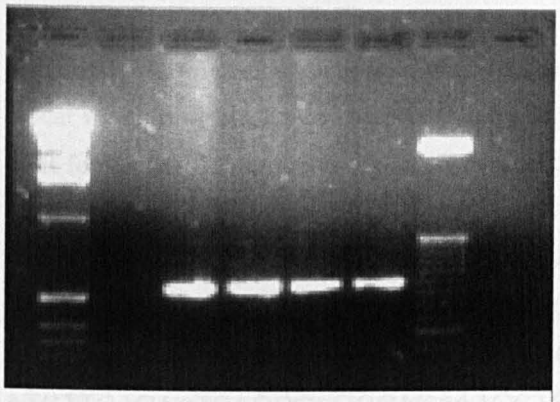


Figure 4.6 Results of PCR to amplify *APC* (wild type) Lane 1: 1kb ladder, lane 2: negative control, lanes 3-6: cell lines and normal DNA, lane 7: 50bp ladder.

PCR products from this reaction were subjected to sequencing analysis with NCBI BLAST and were shown to have the sequence of human *APC*.

We initially attempted to use this DNA in a mismatch ligation assay to detect the two commonest somatic *APC* mutations. These are a single base substitution at codon 1450, and a 5 base pair deletion at codon 1309. This proved unsuccessful, with the MLA for the codon 1450 mutation failing to give a positive result even with a cell line known to have that mutation, and the MLA for the codon 1309 mutation giving false positive results. With hindsight, the PCR product was too long for satisfactory MLA results, and the nature of the codon 1309 mutation (loss of a repeated sequence) contributed to the poor results.

Because of these problems, we decided to use mutant allele-specific PCR to look for the mutation at codon 1309. This mutation is a 5bp deletion of a repeated AAAAG sequence.

The sense primer was designed to have the mutation site close to its 3' end. Because the deleted bases are part of a repeated sequence, it was not possible to have the mutation at the end of the primer. The anti-sense primer was the same as that used to amplify wild type *APC*.

Figure 4.7 shows the sequence of part of the *APC* gene. The original sense and anti-sense primers for the amplification of wild type *APC* are highlighted, as is the five base pair repeat which is deleted in codon 1309 mutations. The sense primer for mutation specific PCR for codon 1309 mutations is also indicated.

GACGACACAGGAAGCAGATTCTGCTAATACCCTGCAAATAGCAGAAATAAAA
G(AAAAG)ATTGGAACTAGGTCAGCTGAAGATCCTGTGAGCGAAGTTCCAGC
AGTGTCACAGCACCCTAGAACCAAATCCAGCAGACTGCAGGGTTCTAGTTT
ATCTTCAGAATCAGCCAGGCACAAAGCTGTTGAATTTTCTTCAGGAGCGAAA
TCTCCCTCCAAAAGTGGTGCTCAGACACCCAAAAGTCCACCTGAACACTAT
GTTCAGGAGACCCCACTCATGTTTAGCAGATGTACTTCTGTCAGTTCACTTG
ATAGTTTTGAGAGTCGTTCGATTGCCAGCTCCGTTCAGAGTGAACCATGCAG
TGGAATGGTAAGTGGCATTATAAGCCCCAGTGATCTTCCAGATAGCCCTGG
ACAAACCATGCCACCAAGCAGAAGTAAAACACCTCCACCACCTCCTCAAAC
AGCTCAAACCAAGCGAGAAGTACCTAAAAATAAAGCACCTACTGCTGAAAAG
AGAGAGAGTGGACCTAAGCAAGC

Figure 4.7 Part of the sequence of the *APC* gene, showing position of primer sequences. Primers to amplify wild type *APC* are highlighted in red, the 5bp deletion found at codon 1309 in some colorectal tumours is bracketed and highlighted in green and the sense primer for mutant allele specific PCR consists of the bases highlighted in yellow.

The optimisation of the mutation specific PCR was performed in two stages. The aim of the first stage was to ensure that a positive result was obtained with mutant, but not with wild type DNA. The colorectal cancer cell line LS1034 has been shown to have the relevant mutation and DNA from these cells was used as a positive control. Each reaction also included 2 negative controls; one tube with no DNA as a control for contamination, and one tube containing DNA from a healthy volunteer, to check for specificity.

Conditions were made more stringent (higher annealing temperature, lower magnesium concentration) until this was achieved. An annealing temperature of 59°C and a magnesium concentration of 2mM were found to produce clear results in the positive but not the negative controls.

PCR products from normal DNA and from LS1034 (mutant DNA), amplified using the 'wild type' *APC* primers, were submitted for sequencing analysis. The results of sequencing normal and mutant *APC* are shown in figures 4.8 and 4.9 respectively.

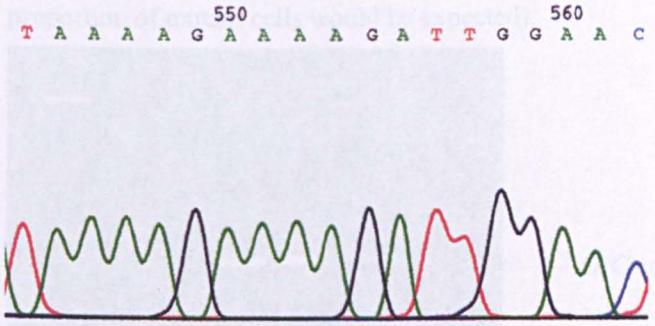


Figure 4.8 Sequencing result from tumour with wild type *APC* at codon 1309. The 5 base pair repeated AAAAG sequence is seen, beginning at base 545.

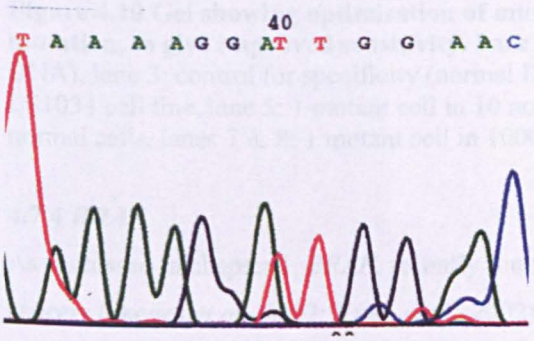
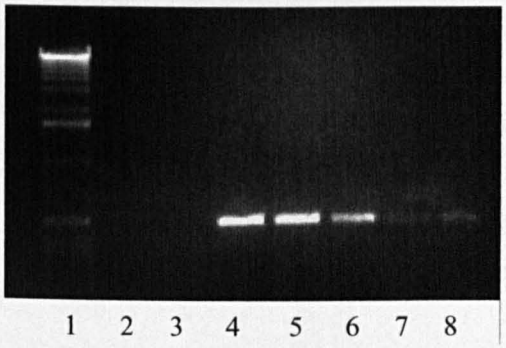


Figure 4.9 Sequencing result from LS1034 cell line, with 5 base pair deletion at codon 1309 of *APC*. Loss of the repeated AAAAG is seen.

The second stage of the optimisation was necessary to ensure that the test was sensitive enough to detect low levels of mutant DNA on a background of normal DNA. A series of samples were prepared containing 10^6 normal cells mixed with 10^5, 10^4 and 10^3 LS1034

cells, giving a concentration of one mutant cell in ten, one hundred and one thousand respectively. With the conditions described above it was only possible to detect one mutant cell in one hundred normal cells. A number of experiments were performed to balance the need for sensitivity against the need for specificity. An annealing temperature of 57°C, with a magnesium concentration of 2.5mM allowed one mutant cell in 1000 to be detected, with no amplification of wild type DNA. These results are illustrated in figure 4.10. As described in chapters 5 and 6, the more stringent PCR conditions were used to examine DNA from primary tumours, with the more sensitive conditions being used to examine peritoneal samples from the same patients (where a much lower proportion of mutant cells would be expected).



← *APC* codon 1309 mutant PCR product

Figure 4.10 Gel showing optimisation of mutation specific PCR for *APC* codon 1309 mutation, to give improved sensitivity. Lane 1: 1kb ladder, lane 2: negative control (no DNA), lane 3: control for specificity (normal DNA), lane 4: positive control (DNA from LS1034 cell line, lane 5: 1 mutant cell in 10 normal cells, lane 6: 1 mutant cell in 100 normal cells, lanes 7 & 8: 1 mutant cell in 1000 normal cells.

4.7.4 *BRAF*

As discussed in chapter 1, *BRAF*, recently found to be mutated in about 10% of colorectal cancers (Davies *et al.*, 2002; Yuen *et al.*, 2002) was a good target for detection in our study. Tumours with the commonest *BRAF* mutation, a T→A mutation at codon 599 do not have *K-ras* mutations. This meant that we were likely to be detecting mutations in tumours in which we had not found a mutation previously, rather than detecting second mutations in tumours. We decided to use a Mismatch Ligation Assay to detect these mutations because we were familiar with this technique, and it was applicable to both tumour DNA and peritoneal samples.

The first step in designing an MLA to detect *BRAF* mutations was to amplify the area of DNA containing the mutation, using PCR. The optimal size of PCR product for MLA is less than 100 base pairs (K. Hibi, personal communication). For this reason, the primers described by Davies (supplementary information to paper, given on Nature website, http://www.nature.com/cgi-taf/DynaPage.taf?file=/nature/journal/v417/n6892/full/nature00766_fs.html), which give a product 224 base pairs long, were not suitable. The DNA sequence of *BRAF* exon 15 was obtained from the website of the Sanger Institute (http://www.ensembl.org/Homo_sapiens/geneview?gene=ENSG00000157764), and Primer Designer software (Scientific & Educational Software, Durham, NC, USA) was used to select a pair of primers to amplify the relevant portion of exon 15, without overlapping the mutation site. It proved difficult to design an ideal set of primers, and the subsequent PCR was prone to primer dimer formation. Reaction conditions were optimised, and these are detailed in the appendix. An example of a PCR to amplify *BRAF* is shown in figure 4.11.

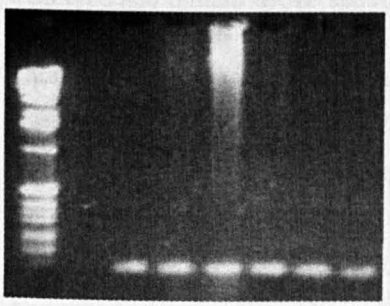


Figure 4.11 Gel showing PCR to amplify short section of *BRAF* exon 15
Lane 1: 1kb ladder, lane 2: negative control (no DNA), lanes 3-8: various tumour DNAs.

4.8 Mismatch Ligation Assays (MLA)

4.8.1 Oligonucleotide design and reaction conditions

Details of oligonucleotides and reaction conditions for MLA to detect *K-ras* and *TP53* mutations were available in the literature (Dong *et al.*, 2001; Jen *et al.*, 1994b). These

details are listed in the appendix. For the MLA to detect mutant *BRAF*, oligonucleotides were designed, using the principles apparent in the literature, and described in section 2.7.1. These sequences are shown, in alignment with the wild type and mutant sequences, in figure 4.12.

Wild type *BRAF* sequence

GTCTAGCTACAGTGAAATCTCGATGGAGT

Blocking oligonucleotide (complementary to wild type sequence on opposite strand)

ACAGTGAAATC

Mutant *BRAF* sequence (mutation highlighted in red)

GTCTAGCTACAGAGAAATCTCGATGGAGT

Mutation specific oligonucleotide (BRAF 5)

TAGCTACAGA

Nucleotide for 5' end-labelling (BRAF 3)

GAAATCTCGA

Figure 4.12 Oligonucleotides for *BRAF* MLA. These are shown in alignment with wild type and mutant *BRAF* sequences.

Three of the cell lines available in our laboratory have been shown to have the relevant point mutation in *BRAF* (COLO 205, HT 29 and SW1417). These were used as positive controls. DNA from healthy volunteers was initially used as a negative control, and later DNA from a tumour shown to be wild type on MLA testing (and confirmed by direct sequencing). An example of an MLA showing that mutant and wild type *BRAF* can be distinguished using these oligonucleotides is shown in figure 4.13.

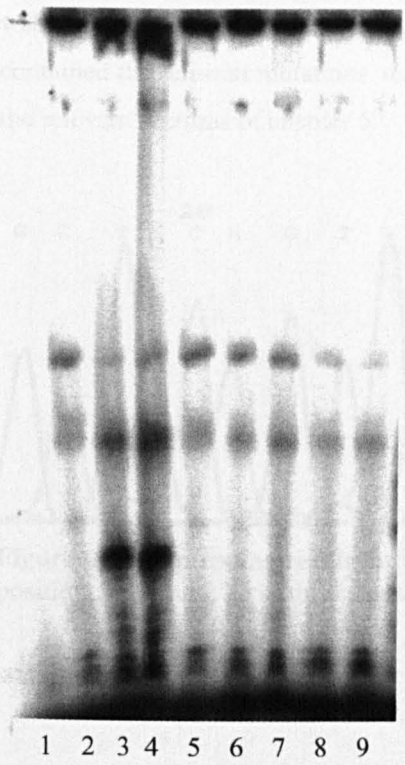


Figure 4.13 Example of MLA to detect mutant *BRAF*. Lane 1: No DNA, lane 2: PCR product from DNA of healthy volunteer, lane 3: HT 29, lane 4: COLO 205, lanes 5-9: tumours 1-5. A clear band is seen in both tumour lines but not in negative controls, or these tumours.

MLA was optimised using conditions similar to those found to be appropriate for other reactions. 200ng of blocking oligonucleotide and 25ng of mutation specific oligonucleotide per reaction gave the clearest results. Full details of the reaction conditions are given in the appendix.

The presence of the expected mutation in the positive control cell line, and the lack of a mutation in the negative control tumour sample were confirmed by sequencing. PCR products for the sequencing reaction were prepared using the exon 15 primers described previously (Davies *et al.*, 2002, see website address above). The longer PCR product amplified by these primers was more suitable for sequencing analysis. PCR products were purified prior to sequencing using a PCR Purification Kit (Qiagen), used according to the manufacturers' instructions. Results are shown below, in figures 4.14 and 4.15. It

was also possible to confirm that tumours giving positive results with the other MLAs contained the relevant mutations, using direct sequencing. Examples of this are given in the relevant sections of chapter 5.

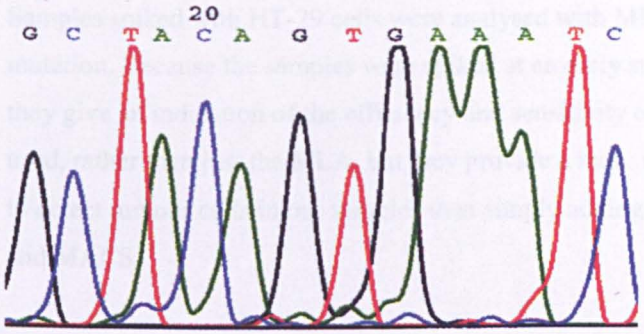


Figure 4.14 Sequencing result for tumour 1. This shows wild type *BRAF* with T at position 1796 (base 24 in this sample). The sequence shown is GCTACAGTGAAATC.

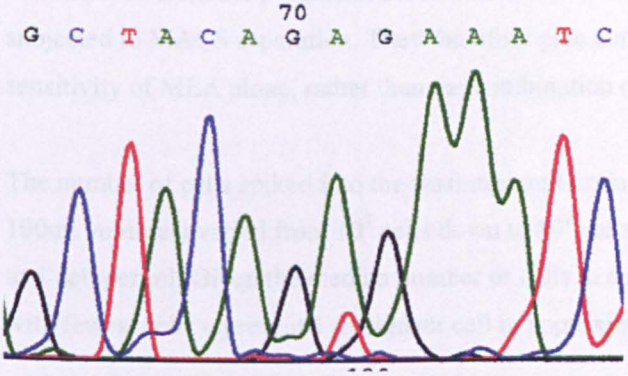


Figure 4.15 Sequencing result for COLO205 cell line. The sequence shown is GCTACAGAGAAATC. This confirms a T→A mutation at position 1796 (base 71 in this sample).

4.8.2 Assessment of Sensitivity of MLA

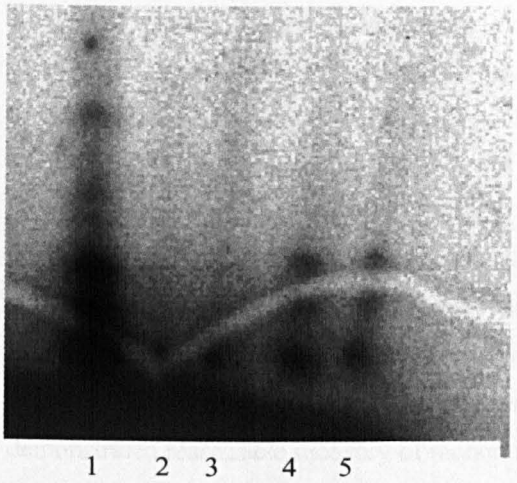
The sensitivities of the various MLA reactions were assessed using spiked samples. Samples to assess *K-ras* and *TP53* assays were prepared, containing varying numbers of cells from colorectal cancer cell lines. These cells were introduced into washings and drain fluid samples from patients undergoing surgery for benign diseases. The cancer cells were introduced as soon as the samples were brought to the laboratory, and the

samples were then subjected to density centrifugation and MACS separation, with the pellets being frozen down. DNA was extracted from the pellets, and the appropriate exon amplified by PCR. PCR products were used in a MLA. Samples spiked with SW480 cells could be analysed with MLA for both *K-ras* codon 12 and *TP53* codon 273 mutations. Samples spiked with HT-29 cells were analysed with MLA for the *TP53* codon 273 mutation. Because the samples were spiked at an early stage in the process of analysis they give an indication of the efficiency and sensitivity of the combination of techniques used, rather than just the MLA, but they provide a more realistic assessment of our ability to detect tumour cells in our samples than simply adding cells after density centrifugation and MACS.

The MLA to detect *BRAF* mutations was assessed for sensitivity using samples that were prepared by adding known numbers of cells from the COLO 205 cell line to mononuclear cells derived from the peripheral blood of healthy volunteers. These samples were not subjected to MACS separation. They therefore give a more accurate estimation of the sensitivity of MLA alone, rather than the combination of techniques.

The number of cells spiked into the washings and drain fluids (which were normally 100ml volumes) varied from 10^5 cells down to 10^2 cells i.e. from 1000 cells per ml down to 1 cell per ml. Given the median number of cells in our samples (1.6×10^7), the samples with fewest cells represent 1 malignant cell in approximately 10^5 normal cells.

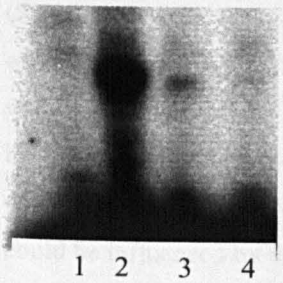
Considering those samples assessed with the *K-ras* MLA, mutant DNA could be detected in 2 of the 4 samples spiked with 10^3 cells but in neither of the samples spiked with 10^2 cells. The *TP53* MLA is possibly more sensitive. Both of the samples spiked with 10^5 cells were clearly positive, one of the 4 spiked with 10^3 cells, and 2 of the 3 spiked with 10^2 cells. An example of this is shown in figure 4.16.



←position of band in presence of *TP53* mutation

Figure 4.16 Results of spiked samples in p53 MLA. The first lane shown demonstrates a positive result in tumour 28. Lanes 4 and 5 show positive results from samples spiked with 10^3 and 10^2 SW480 cells respectively.

As explained above, the *BRAF* MLA was assessed using a different type of sample. Samples were prepared containing one mutant cell in 30 to 3200 normal cells, and each sample was analysed at least twice. Clear positive results were shown with the sample containing one mutant cell in one thousand normal cells. An example of this is shown in figure 4.17.



← position of band in presence of *BRAF* mutation

Figure 4.17 Results of spiked samples in *BRAF* MLA. Lane 1: negative control, lane 2: positive control, lane 3: 1 mutant cell in 1000 normal cells, lane 4: 1 mutant cell in 3200 normal cells.

In order to reduce concerns about possible false positive results when using MLA to test peritoneal samples, washings and drain fluids from 4 patients undergoing surgery for benign diseases were subjected to various MLA tests, and all gave negative results.

4.9 Discussion

As far as possible, we have attempted to validate each section of our experimental strategy, with quantification of our results wherever possible. Each experiment has incorporated appropriate positive and negative controls, with the negative controls for the MLA reactions including the relevant PCR product rather than no DNA. We have demonstrated reasonable recovery of mononuclear cells after density centrifugation and that losses during the MACS process were at an acceptable level. We have successfully obtained amplifiable DNA from almost all our samples, despite the small number of cells in many of them. We have been able to confirm that our PCR and MLA reactions are amplifying and detecting the appropriate areas or mutations in DNA by sequencing analysis.

A few questions remain about the sensitivity of MLA. The results from the BRAF samples suggest that the technique, when used alone, is able to detect one mutant cell in one thousand normal cells. This is consistent with results reported in the literature (Sanchez-Cespedes *et al.*, 1999). The results from the samples subjected to density centrifugation and MACS are less clear-cut. Although positive results could be obtained with one mutant cell per millilitre of initial sample in some cases, these results could not be repeated in a number of later samples. This variability may be due to the large number of different processes needed to produce the final MLA result. It is possible that results could be influenced by the use of different batches of antibodies or by the change in supplier of many of the enzymes used for these reactions during the course of this research.

CHAPTER 5: DETECTION OF MUTATIONS IN PRIMARY TUMOUR SAMPLES

5.1 Introduction

As discussed in section 1, we elected to use DNA based techniques to search for cancer cells in the peritoneal samples from our patients. The disadvantage of DNA based techniques for detection of small numbers of colorectal cancer cells is the lack of a single mutation that occurs in all or even most colorectal tumours. This means that it is necessary to look for mutations in a number of different genes (and different mutations within those genes) in order to assess the full range of tumours. It is therefore not practical to test samples for minimal residual disease for all possible colorectal cancer associated mutations, whether the samples are bone marrow, lymph nodes or, in this case, peritoneal washings and post-operative drain fluids. In order to reduce the number of tests needed, it is first necessary to determine whether a particular mutation is present in the primary tumour from a given patient. When a mutation is detected the other samples from that patient are examined to determine whether the same mutation is present in cellular material, indicative of the presence of cancer cells.

This chapter describes the results of our experiments to detect mutations in the DNA from our primary tumour samples. As discussed in chapter 1, we decided to use the same techniques to look for mutations in the primary tumours and then in the peritoneal samples. However, where possible, the presence of the mutations detected by these techniques was confirmed by sequencing analysis.

The choice of mutations as targets for detection is discussed in chapter 1 and sections 4.7.3 and 4.7.4.

5.2 Sample Collection and Processing

Samples of 46 primary tumours were collected as described in section 2.2 and stored at -80°C until needed. DNA was extracted from the tumours after grinding under liquid nitrogen (section 2.4.1). The concentration of DNA extracted was quantified for the first

19 tumours using a spectrophotometer. The mean DNA concentration was 92ng/μl (range 13- 210ng/μl). Later samples were not tested as all tumours yielded amplifiable DNA; however, the DNA from three tumours sometimes failed to amplify at the first attempt.

5.3 *K-ras* Mutations

Exon 1 of *K-ras* was amplified in a PCR reaction: typical results are shown in figure 4.4. We were able to amplify this exon from all tumour DNA samples, although sometimes this took several attempts. The products from this PCR were used in a mismatch ligation assay (MLA), the principles of which are described in section 2.7.1. The master mix included the three oligonucleotides complementary to the three possible mutant bases at codon 12B. This allowed us to detect all possible point mutations at this position. A blocking oligonucleotide, complementary to the wild type sequence was included to reduce the risk of false positive results. A common oligonucleotide, complementary to the sequence immediately adjacent to the mutation site, was 5' end labelled using ^{32}P γATP and then added to each sample. Oligonucleotide sequences are given in the appendix.

Each tumour sample was analysed at least twice by MLA, and any sample giving at least one unequivocally positive result was considered to contain a mutation at codon 12B of *K-ras*. 13 tumours from 12 patients had *K-ras* mutations; tumours 36 and 37 were two separate left colonic tumours in the same patient. A gel showing positive results from several of these tumours is shown in figure 5.1. Pictures of other gels showing positive results from the other tumours listed are included in the appendix. As seen in this figure, a typical gel from a *K-ras* MLA experiment gave an appearance of multiple bands in the positive control and in the samples giving positive results. The exact nature of these bands is unclear. The bands close to the solvent front were seen repeatedly in positive samples, and have been noted by others using MLA (K Hibi, personal communication). They are not seen in the negative control samples, which, as explained previously, contain PCR product of the appropriate exon from a healthy volunteer. The larger bands, towards the top of the gel, probably represent residual double stranded DNA, where radiolabelled MLA product is annealed to template PCR product. These bands were

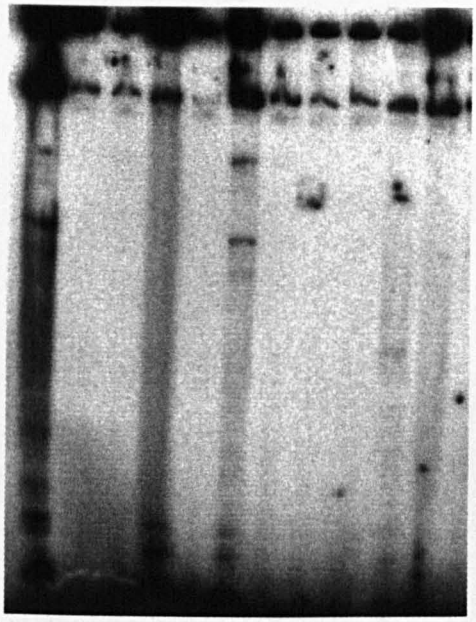
sometimes seen in negative control lanes, and cannot therefore be considered to show positive results.

We attempted to confirm our positive results by sequencing analysis. Primers amplifying a longer stretch of the *K-ras* gene (389bp, including exon 1) were used for this purpose. Primer sequences and reaction conditions are given in the appendix. An example of a G→A mutation at codon 12B is shown in figure 5.2. The sample contained a mixture of cells with the mutation and with the wild type sequence, giving the double peak shown.

The characteristics of the tumours giving positive results are shown in table 5.1, along with the results of sequencing analysis for each tumour sample.

TUMOUR NO.	DUKES' STAGE	TNM STAGE	SEQUENCING RESULT
2	B	T3N0MX	G→A
4	ADENOMA		G→A
13	B	T3N0MX	G→A
20	C1	T2N2MX	NO RESULT
23	RECURRENT DISEASE		G→A
26	B	T4N0MX	G→A
29	B	T3N0MX	G→A
31	B	T4N0MX	G→C
32	B	T3N0MX	WILD TYPE
36	D	T3N1M1	WILD TYPE
37	D	T3N1M1	WILD TYPE
42	D	T4N1M1	WILD TYPE
49	A	T1N0MX	G→A

Table 5.1 Characteristics of tumours with K-ras mutations, and results of sequencing analysis.



1 2 3 4 5 6 7 8 9 10 11

Figure 5.1 Example of gel showing positive result for K-ras mutations in tumour samples. K-ras experiment 28, showing positive results in lanes 1, 4, 6, 10 & 11. Lane 1, positive control (SW480 cell line), lane 2, negative control (K-ras PCR product from healthy volunteer), lane 3 tumour 1, lane 4 tumour 2, lane 5 tumour 3, lane 6 tumour 4, lanes 7-9 tumours 9, 10 & 14, lane 10 tumour 20, lane 11 tumour 23.

The four tumours, which gave a wild type result on sequencing, may represent false positive results from the MLA tests. Given the use of stringent negative controls in all MLA experiments, however, it is perhaps more likely that they represent tumours in which only a fraction of the cells from which DNA was isolated contained the relevant *K-ras* mutation. Sequencing may well not detect mutant DNA against a background of excess wild type DNA. This will always be present in a tumour, especially when the oncogene mutation is dominant, and so cancer cells are likely to be heterozygous (as is the case with *K-ras*). It can be seen from the sequencing data in figure 5.2, that even when mutant DNA is clearly seen, a large amount of the wild type base is also present. Tissue cut from a colorectal tumour, without microscopic dissection, will contain stromal cells and blood cells, as well as epithelial cells. It is also possible that not all the epithelial cells in the sample were derived from the cancer, with some possibly coming from

residual adenomatous tissue or adjacent normal mucosa. Even within the cancer cells of a tumour, not all may contain the same mutation.

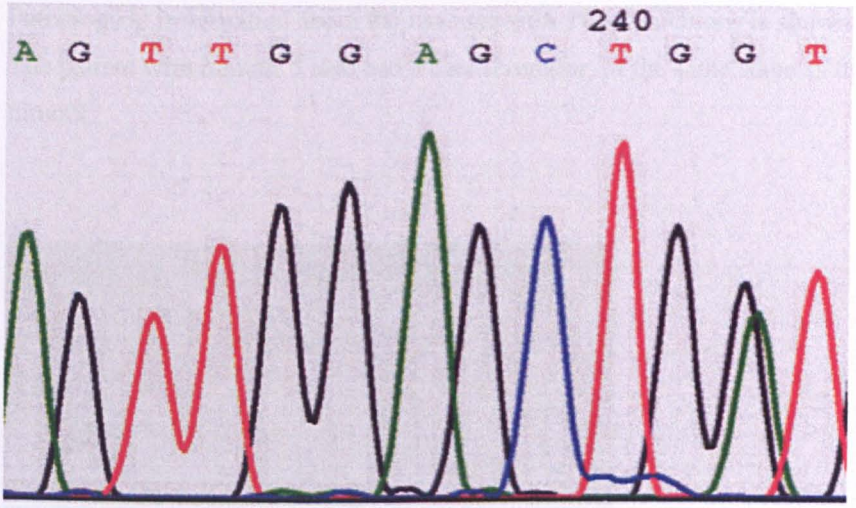


Figure 5.2 Sequencing result from tumour 4 showing the presence of a K-ras mutation. The base at position 242 shows a mixture of G and A. The sample contained some cells with the wild type codon GGT and others with the mutant codon GAT.

5.4 *TP53* mutations

We studied two mutations of exon 8, again using a Mismatch Ligation Assay, as described by Dong to look for exfoliated tumour cells in the faeces (Dong *et al.*, 2001). These mutations involve a change at codon 273 from the wild type codon CGT to TGT or CAT.

Exon 8 of *TP53* was amplified in a PCR using the primers described by Dong. Appropriate sized products could be obtained from all samples; an example is shown in figure 4.5. Each tumour was analysed in a MLA at least twice. Tumour 28 gave positive results on multiple occasions. An example of this is shown in figure 5.3. Tumour 5 gave a single apparently positive result, but this could not be repeated, casting some doubt on the presence of a *TP53* mutation in this tumour. The picture demonstrating this is included in

the appendix. Again, multiple bands are seen in this gel in both the positive control and the tumour with the relevant mutation, but are not present in the negative control sample.

Pathological information about the tumours with *TP53* mutations is shown in table 5.2. The patient with tumour 5 also had a caecal tumour, of the same stage as his sigmoid tumour.

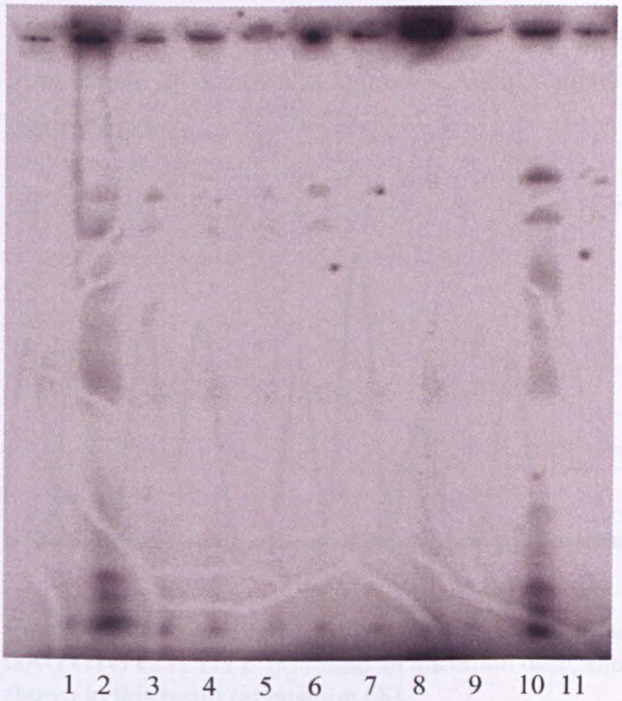


Figure 5.3 Gel picture showing positive result for p53 mutation in tumour 28.
Lane 1 negative control, lane 2 positive control (SW480 cell line), lanes 3-11 tumours 21 to 29 in order. The positive result in lane 10 corresponds to tumour 28.

TUMOUR NO.	DUKES' STAGE	TNM STAGE	SEQUENCING RESULTS
5	B	T3N0MX	WILD TYPE
28	B	T3N0MX	CGT→CAT

Table 5.2 Characteristics of tumours with p53 mutations.

PCR products from these tumours were submitted for sequencing analysis. The result from tumour 28 is shown as figure 5.4. Again, a mixture of wild type and mutant sequence is seen.

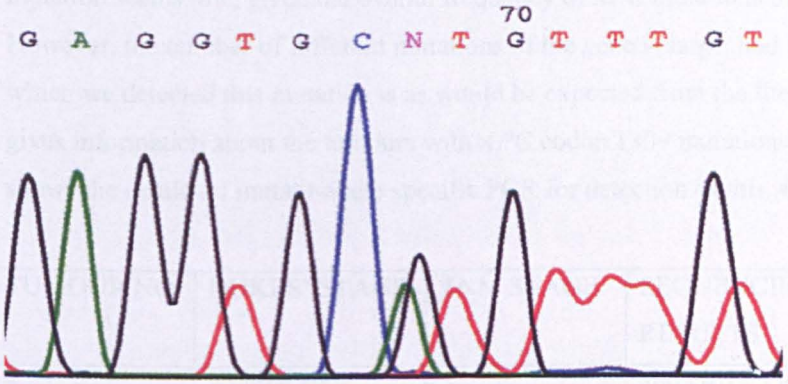


Figure 5.4 Sequencing result from tumour 28. The wild type sequence at this site is GAG GTG CGT GTT TGT, and an uncertain base, showing peaks for both A and G is shown in this result (at position 68).

5.5 *APC* Codon 1309 Mutations

As described in section 4.7.3, attempts to design MLA experiments to detect common *APC* mutations failed. We therefore developed a mutant-allele specific PCR to detect the 5bp deletion at codon 1309. For the detection of mutations in the primary tumour DNAs, we used the more stringent (i.e. more specific) conditions. We would expect a large proportion of the cells in our samples to have the same mutation if it was present in a

particular tumour, and therefore the stringent conditions should allow the truly positive tumours to be detected. Each sample was tested at least twice, and results were consistent across repeated tests. Each reaction contained a negative control with no DNA, a control for specificity containing DNA from a healthy volunteer, and a positive control containing DNA from the LS1034 cell line, which has been shown to have the relevant 5 base pair deletion at codon 1309 (see figure 4.9).

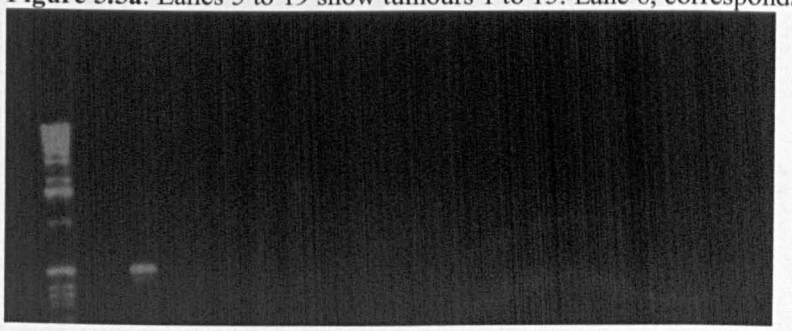
Of the fifty tumours tested, three gave positive results indicating the presence of mutant DNA. It is possible that a mutation could have been missed in one of the tumours whose DNA would sometimes fail to amplify with PCR (particularly numbers 19 and 37) but repeating the test at least twice made this less likely. The proportion of tumours with this mutation seems low, given the overall frequency of *APC* mutations in colorectal cancer. However, the number of different mutations of the gene is large, and the frequency with which we detected this mutation is as would be expected from the literature. Table 5.3 gives information about the tumours with *APC* codon 1309 mutations, and figure 5.5 shows the results of mutant-allele specific PCR for detection of this *APC* mutation.

TUMOUR NO.	DUKES' STAGE	TNM STAGE	SEQUENCING RESULTS
4	ADENOMA		5bp DELETION
45	C1	T3N2MX	5bp DELETION
47	B	T3NOMX	5bp DELETION

Table 5.3 Characteristics of tumours with APC codon 1309 mutations.

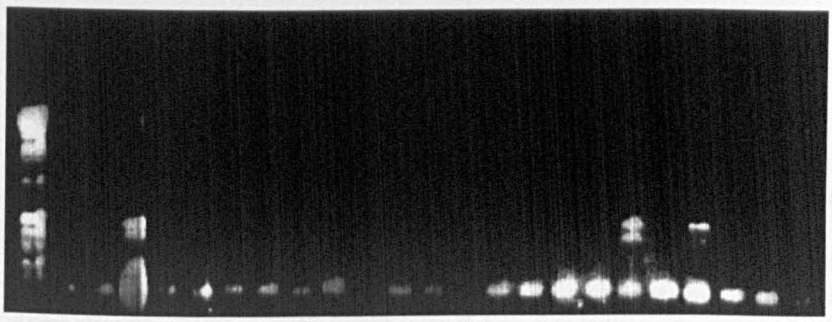
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

Figure 5.5a. Lanes 5 to 19 show tumours 1 to 15. Lane 8, corresponds to tumour 4.



1 2 3 4 18

Figure 5.5b. Tumours 16 to 30 are shown in lanes 4 to 18. There are no positive tumours.



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 19 21 23

Figure 5.5c. Tumours 31 to 50 are shown in lanes 5 to 24. The positive results in lanes 19 and 21 correspond to tumours 45 and 47.

Figure 5.5a, b & c. Gel pictures showing results of mutant–allele specific PCR for APC codon 1309 mutations. In 5.5a and 5.5c lane 1 contains 1kb ladder, lane 2 negative control (no DNA), lane 3 DNA from healthy volunteer and lane 4 positive control. In 5.5b Lane 1 contains ladder, lane 2 negative control and lane 3 positive control.

DNA from the three tumours giving positive results was submitted for sequencing analysis. As described in section 4.7.3, this was amplified using primers designed to produce a product including the mutation site towards the middle of the amplicon (i.e. not the mutation specific primers). The results show a mixture of wild type DNA and mutant DNA, containing a 5 base pair deletion. Because of the position of the primers used, this is most clearly seen on the reverse strand, and therefore the sequence shown is complementary to that given in the previous chapter. Figure 5.6 shows the result of sequencing analysis in tumour 45. The wild type and mutant sequences are first shown, in alignment, with the five base pairs deleted in mutant APC highlighted. These sequences are compared with the result of the sequencing analysis. Up to the point of the mutation, the sequence is clearly seen and unambiguous. From the mutation site onwards, the overlapping mutant and wild type sequences are both seen.

A T C T T T T C T T T T A T T T C T G C T A
A T C T T T T A T T T C T G C T A

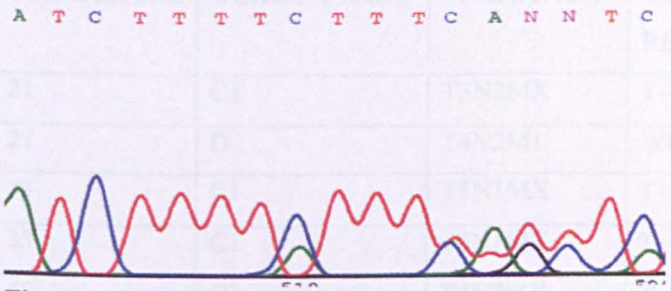


Figure 5.6 *APC* sequence with and without 5bp deletion compared with sequencing result. The first line of sequence shows the wild type sequence (reverse strand) with the 5 bases deleted in the mutant form highlighted in red. The second line shows the mutant sequence, aligned for comparison. The Chromas picture shows the result of sequencing tumour 45, and the expected bases can be seen when this is compared with the sequences given above.

5.6 *BRAF* Mutations

As discussed in section 1 and sections 4.7.4 and 4.8.1, *BRAF* was selected as an appropriate target for mutation detection, and a MLA was designed, which was capable of detecting mutant BRAF. Only one mutation specific oligonucleotide was included in each reaction, because only a T→A mutation has been described at the relevant site.

DNA from 45 of the 46 colorectal tumours was tested for the commonest *BRAF* mutation. DNA from one tumour repeatedly failed to amplify in the appropriate PCR. Each tumour was tested at least twice, and results were consistent across repeated tests. Each MLA experiment included a positive control (PCR product from a cell line known to have the relevant mutation) and a negative control (PCR product from a healthy volunteer, or later from a tumour that had repeatedly given negative results).

Of the 45 tumours tested for *BRAF* mutations, 5 gave positive results. These results were consistent across repeated tests. The characteristics of the tumours with *BRAF* mutations are shown in table 5.4. Figure 5.7 is an example of a gel showing a positive result in tumour 21. Other gel pictures, showing positive results from the other tumours, are shown in the appendix.

TUMOUR NO.	DUKES' STAGE	TNM STAGE	SEQUENCING RESULTS
21	C1	T3N2MX	T→A
27	D	T4N2M1	WILD TYPE
33	C1	T3N1MX	T→A
35	C1	T3N1MX	T→A
48	C2	T4N2MX	T→A

Table 5.4 Characteristics of tumours with *BRAF* mutations.

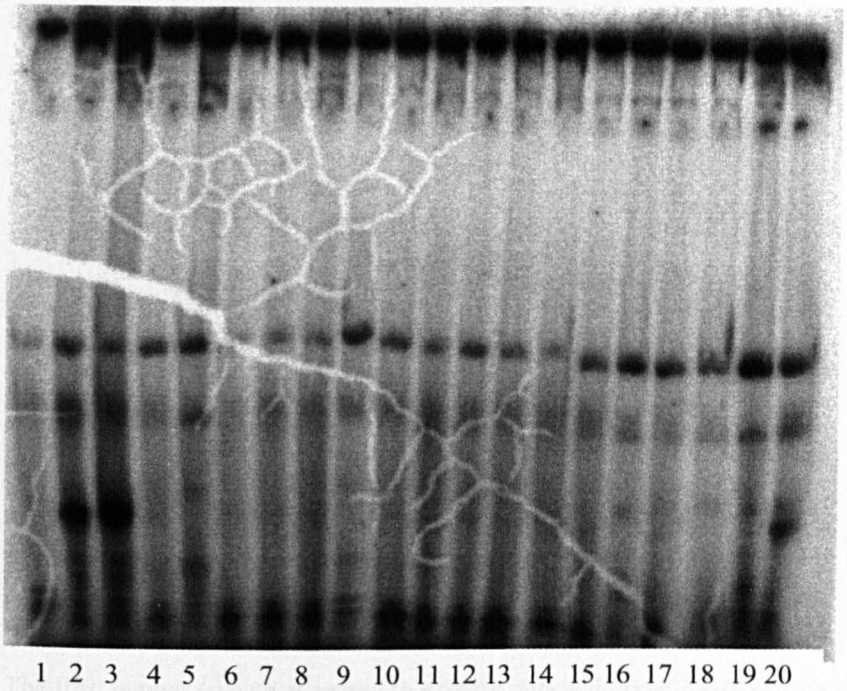


Figure 5.7 showing positive result for *BRAF* mutation in tumour 21. Lane 1 negative control, lanes 2& 3 positive controls, lanes 4-20 tumours 2-7, 9-18 & 21 in order. Positive results are seen in lanes 2, 3 and 20.

All tumours with *BRAF* mutations were at least Dukes' stage C tumours. When comparing tumours with *BRAF* mutations with those in which a *BRAF* mutation was not detected, the *BRAF* mutant tumours were significantly more likely to be Dukes' C or D rather than A or B ($p<0.02$, Fishers exact test). It is interesting that 3 of the tumours were found in the transverse colon. However, *BRAF* mutant tumours were not significantly more likely to be found proximal to the splenic flexure than those without the mutation ($p=0.15$).

The MLA technique has not previously been described for the detection of *BRAF* mutations, so it was particularly important to check the reliability of our results. We therefore submitted samples from the 5 positive tumours for sequencing analysis. As explained in Chapter 4, different primers were used to amplify a product of appropriate

length. Four of the five tumours with mutations detected by MLA had clear evidence of a mutation at the expected site on sequencing analysis. A typical result is shown below, in figure 5.8.

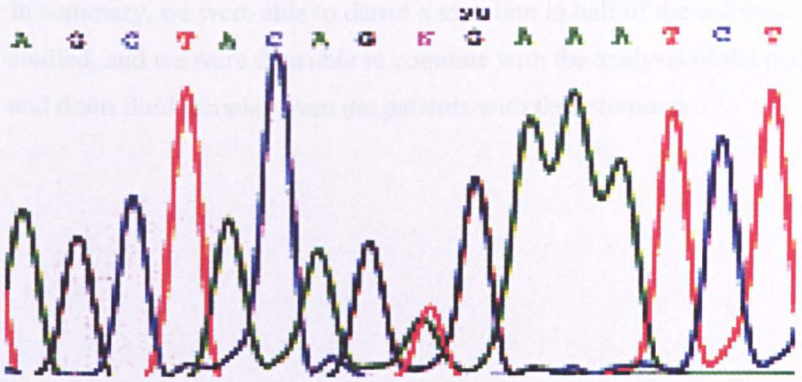


Figure 5.8 Sequencing result from tumour 48 showing codon 599 *BRAF* mutation. This tumour included both normal and mutant DNA giving a mixture of A and T at the relevant site, shown here at position 89.

The fifth tumour (which appeared to contain only wild type *BRAF* on sequencing) is likely to contain a lower level of mutant *BRAF*. Spiking experiments have indicated that using MLA, one mutant cell can be detected among about 1000 normal cells, and, as discussed above, this is a much lower concentration than can be detected using sequencing.

5.7 Discussion

The combination of tests we have described for the detection of mutations in primary tumours has enabled us to detect at least one mutation in 22 of our 46 colorectal tumours. One tumour (a severely dysplastic adenoma) had both a *K-ras* and an *APC* codon 1309 mutation. A tumour with a mutation was found in 21 of the 43 patients studied. Possible ways in which this proportion could be increased are discussed in chapter 8.

The confirmation of the mutation detected by direct sequencing in 16 out of 23 cases is reassuring. As discussed above, the remaining tumours may be those in which a relatively low proportion of the DNA sampled contained the relevant mutation. For example, the

tumour with a *BRAF* mutation, detected on MLA but not on sequencing, gave a consistently weaker (although still clearly positive) band on the MLA gels.

In summary, we were able to detect a mutation in half of the colorectal tumours we studied, and we were then able to continue with the analysis of the peritoneal washings and drain fluid samples from the patients with these tumours.

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Chapter 6: DETECTION OF MUTATIONS IN PERITONEAL WASHINGS AND POST-OPERATIVE DRAIN FLUIDS

6.1 Introduction

Once we had determined which mutations were present in which tumours, we could attempt to answer our initial question about the presence of tumour cells in the peritoneal cavity at various times during and soon after surgery. As described in chapter 2, peritoneal washings were collected from all patients on opening the abdomen, and again after removal of the tumour. We also collected fluid from the abdominal drain, when placed, on the first and second post-operative day. All these samples were subjected to density centrifugation, and MACS was performed either on the whole sample, or in some cases half of the sample, with the remainder being left without separation. These split samples are considered further in Chapter 7. For the remainder of this chapter the results from the separated and non-separated halves of the sample are combined, and reported as positive if either gave a positive result.

6.2 *K-Ras* Mutations

All available peritoneal washing and drain fluid samples, from patients with *K-ras* mutations in their primary tumours, were tested for the presence of the same mutation using the MLA. Each sample was tested at least twice using independent PCR products. The same conditions and control samples were used as when testing the tumour samples. A single clearly positive result was considered to indicate the presence of mutant DNA in that sample.

A median of 3 samples was available for each patient whose tumour was shown to have a *K-ras* mutation, but it should be noted that tumours 36 and 37 are from the same patient and therefore have the same results in the peritoneal samples. Most of the 'unavailable' samples occurred because a drain was not placed (3 patients (4 tumours), 6 missing samples), the wrong type of drain was used (suction drain with cell lysis, one patient), the drain stopped draining (1 sample), or the samples were used to develop techniques (paired samples of tumour 2).

The results of testing the washings and drain fluids from patients with *K-ras* mutations in their primary tumours are shown in table 6.1. The 'overall' column refers to whether any peritoneal sample from that patient gave a positive result.

TUMOUR NUMBER	DUKES' STAGE	PRE-OP	POST-OP	DAY 1	DAY2	OVERALL
2	B			POS		POS
4	ADENOMA	NEG	NEG	NEG	NEG	NEG
13	B	POS	POS			POS
20	C1	NEG	NEG	NEG	NEG	NEG
23	RECURRENCE	POS	POS			POS
26	B		NEG	NEG		NEG
29	B	POS	NEG	POS	NEG	POS
31	B	POS	POS	POS		POS
32	B	POS	POS	POS	POS	POS
36 & 37	D	POS	POS			POS
42	D	POS	POS	NEG		POS
49	A	POS	POS			POS

Table 6.1. Results of peritoneal washings and drain fluids from patients with *K-ras* mutations in their primary tumours. POS = positive result, NEG = negative result. Cells shaded grey represent no sample available for analysis, empty cell indicates amplifiable DNA not obtained. PRE-OP = wash at start of operation, POST-OP = wash at end of operation, DAY 1 = drain fluid from first post-operative day, DAY 2 = drain fluid from second post-operative day, OVERALL, POS = any peritoneal sample positive, NEG= all peritoneal samples negative.

As can be seen from the table, of the 12 patients with a *K-ras* mutation at least one washing or drain fluid was positive in 9 (75%). In fact, all but one of these patients had multiply positive samples. Of the three patients with no evidence of mutant DNA in the peritoneum one had a severely dysplastic adenoma, one a Dukes' B tumour (which was however a T4 lesion) and one a Dukes' C1 tumour (T2).

Of the 9 patients with positive results, suggesting that cancer cells had been shed into the peritoneum, 2 patients (with tumours numbered 36/37 and 42) had proven metastases to the liver, and the patient with tumours 36 and 37 also had local involvement of the bladder. Another patient was undergoing surgery for disease that had already recurred locally (tumour number 23). Details of the remaining patients are discussed in section 6.5.

It can also be seen from the table that in all cases where any washing or drain fluid contained mutant DNA the wash at the start of surgery was always involved. Only two patients showed a mixture of positive and negative results over the time course studied.

An example of a gel showing positive results from peritoneal samples is shown in figure 6.1, other examples are shown in the appendix.

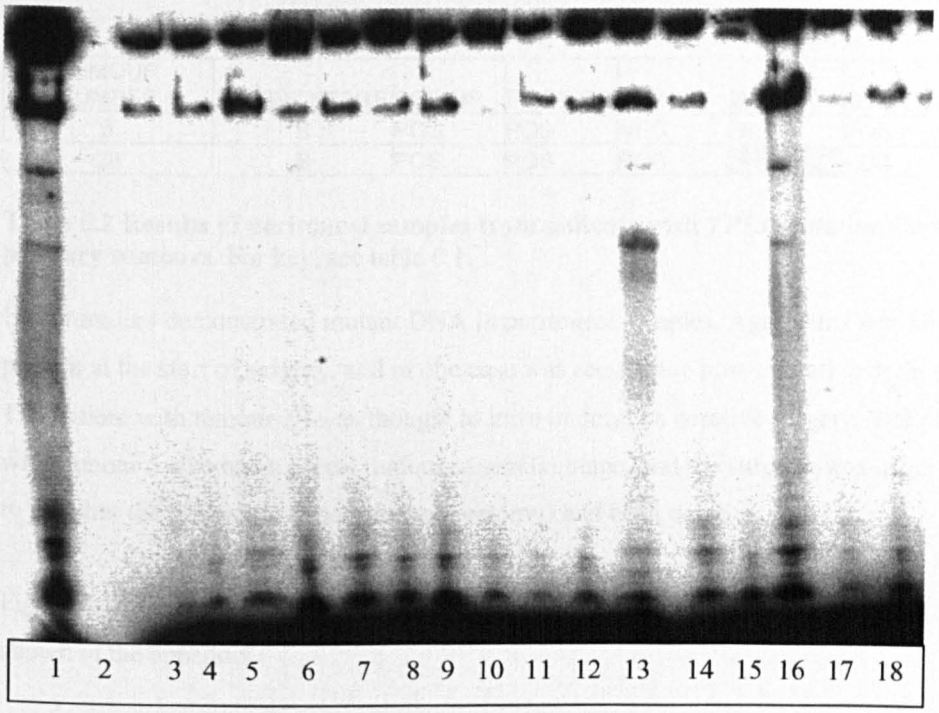


Figure 6.1 Gel from experiment 23, showing positive results in peritoneal samples from patient with tumours 29, 49 and 31. Lane 1 positive control, lane 2 empty, lane 3 negative control. Lane 6 tumour 29, with pre-operative wash and day one drain fluid in lanes 7 & 8 both positive. Lane 9 tumour 49, with positive results in lanes 10 and 12 representing non-separated pre-operative wash and separated post-operative wash respectively. Lane 13 tumour 31, with positive results from non-separated pre-operative wash (lane 14), non-separated post-operative wash (lane 15), separated post-op wash (lane 16) and separated day 1 drain fluid (lane 18).

6.3 *TP53* Mutations

Peritoneal samples from the two patients with colorectal tumours who had *TP53* mutations in their primary tumour were tested for the presence of *TP53* mutations using MLA. In total seven samples, all having been separated using MACS, were available; no fluid drained between the first and second day in the patient with tumour 28. All samples were tested at least twice, and any sample giving a single clearly positive result was treated as positive. The results are shown in table 6.2.

TUMOUR NUMBER	DUKES' STAGE	PRE-OP	POST-OP	DAY 1	DAY2	OVERALL
5	B	POS	POS	NEG	NEG	POS
28	B	POS	POS	POS		POS

Table 6.2 Results of peritoneal samples from patients with *TP53* mutations in their primary tumours. For key, see table 6.1.

Both tumours demonstrated mutant DNA in peritoneal samples. Again, this was always present at the start of surgery, and in one case was seen in the post-operative drain fluid. The patient with tumour 28 was thought to have undergone curative surgery. The patient with tumour 5 also had a caecal tumour of similar stage, and the surgeon was uncertain as to whether the procedure (a panproctocolectomy) had been curative.

Figure 6.2 gives an example of a gel showing positive results, further examples are shown in the appendix.

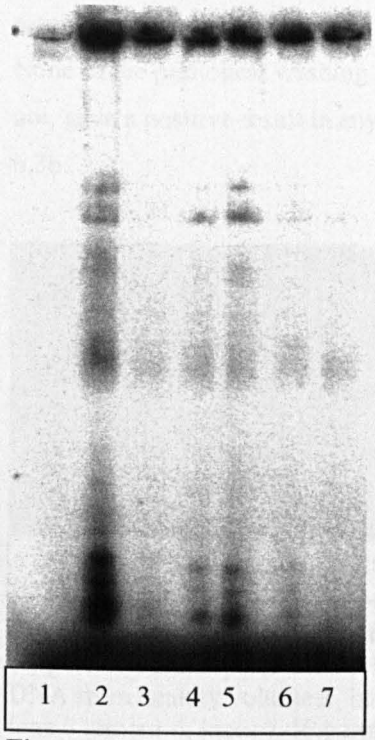


Figure 6.2 Gel picture showing results of MLA of peritoneal samples from tumour 5. Lane 1 negative control, lane 2 positive control, lane 3 tumour 5, lanes 4-7 peritoneal samples from patient with tumour 5 (pre-op, post-op, day 1, day 2 in order.) Positive results seen in lanes 2, 4 and 5.

6.4 *APC* Codon 1309 Mutations

As described in section 5.3.3, three of the fifty tumours had *APC* codon 1309 mutations. Four peritoneal samples were available from tumour 4, and four pairs of samples (half non-separated and half positive fraction after MACS separation) for each of tumours 45 and 47. The samples were assessed for the presence of amplifiable DNA using PCRs for *K-ras* or wild type *APC*, and three proved unreliable. In each case the other half of the sample (2 non-separated, 1 separated) was available and contained easily amplifiable DNA.

The peritoneal washing and drain fluid samples were tested using the less stringent conditions described in section 4.7.3, which gave positive results with a dilution of one tumour cell in 1000 normal cells. Each sample was tested at least twice, with the tumour

sample from the relevant patient included in the reaction as an additional positive control. None of the peritoneal washing or drain fluid samples, whether separated by MACS or not, gave a positive result in any test. These results are illustrated in figures 6.3a and 6.3b.

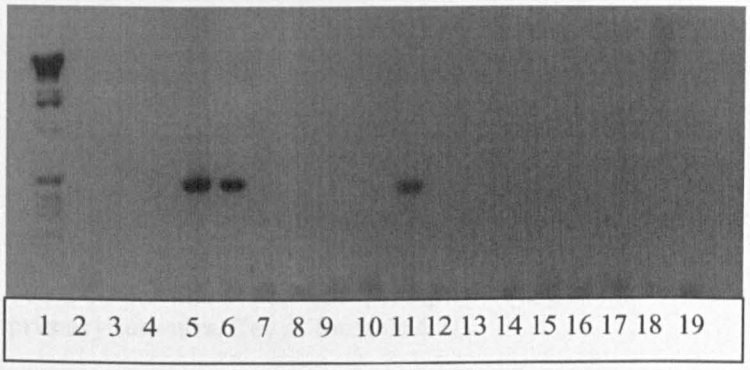


Figure 6.3a Gel showing results of allele- specific PCR for *APC* codon 1309 mutation on peritoneal samples. Lane 1 1kb ladder, lane 2 negative control (no DNA), lane 3 DNA from healthy volunteer, lane 4 empty, lane 5 positive control (LS1034 cell line), lane 6 tumour 4, lanes 7-10 peritoneal samples from patient with tumour 4, lane 11 tumour 47, lanes 12-19 peritoneal samples from patient with tumour 47.

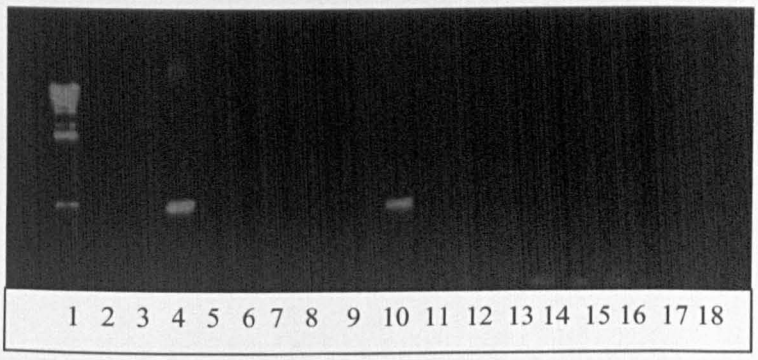


Figure 6.3b PCR for *APC* codon 1309 mutation. Lane 1 1kb ladder, lane 2 negative control, lane 3 normal DNA, lane 4 positive control, lanes 5-9 other tumours without relevant mutation, lane 10 tumour 45, lanes 11-18 peritoneal samples from patient with tumour 45.

6.5 *BRAF* Mutations

As shown in section 5.3.4, five of the 45 colorectal tumours tested had *BRAF* mutations. In total, 15 peritoneal samples were available from these patients. Amplifiable DNA was

available from all samples. In one case a drain was not placed, and in three other cases it was not possible to collect drain fluid on the second post-operative day. All samples were tested for the presence of mutant *BRAF* using an MLA, and no sample was classified as negative until it had been tested at least twice. The results of testing the peritoneal samples are shown in table 6.3.

TUMOUR NUMBER	DUKES' STAGE	PRE-OP	POST-OP	DAY 1	DAY2	OVERALL
21	C1	POS	NEG	NEG	NEG	POS
27	D	NEG	POS	NEG		POS
33	C1	POS	POS			POS
35	C1	POS	NEG	POS		POS
48	C2	POS	NEG	NEG		POS

Table 6.3 Results of peritoneal samples from patients with *BRAF* mutations in primary tumours. Key as for table 6.1.

It can be seen from the table that all patients with a *BRAF* mutation had at least one positive peritoneal washing or drain fluid. This group contains the only patient with a positive overall result, but with a negative wash at the start of operation; this woman had a very locally advanced tumour and liver metastases and therefore was already known to have incurable disease. In the case of tumours 21 and 48, the initial positive result appears to have been cleared by surgery, although the results of conventional staging mean that both patients would have been offered adjuvant treatment if fit.

An example of a positive result is shown in figure 6.4.

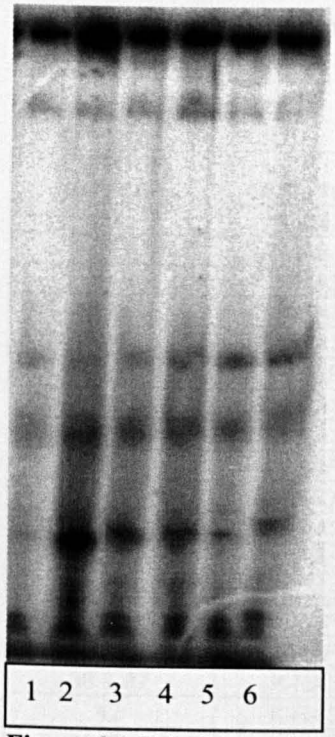


Figure 6.4 Gel picture showing positive result for *BRAF* in peritoneal samples. Lane 1 negative control, lane 2 positive control (cell line DNA), lanes 3-6 peritoneal samples from patient with tumour 33 (pre-op non-separated, pre-op separated, post-op non-separated, post-op separated). Positive results are seen in lanes 2-6.

6.6 Discussion

It has been possible to test peritoneal samples from 21 patients undergoing surgery for colorectal disease. These patients had 22 tumours in which we detected 23 mutations. Positive results have been obtained from at least one peritoneal washing or post-operative drain fluid in 16 patients. The results are summarised in table 6.4.

It is interesting to note the different rates of positive results in patients with different mutations in their primary tumours; *K-ras*, 9 of 12, *TP53*, 2 of 2, *APC* 0 of 3, *BRAF* 5 of 5. This could represent different sensitivities in detection of cells with the different mutations, although both MLA and allele-specific PCR gave positive results at a ratio of one mutant cell to 1000 normal cells in spiking experiments. It is possible that the differences could represent differences in behaviour between tumours with different mutations, or be due to the different stages in malignant transformation at which various

mutations occur, but with such small numbers of patients, any differences may well be due to chance.

TUMOUR NUMBER	MUTATION	OVERALL	DUKES'	TNM
2	*K-RAS*	POS	B	T3N0MX
4	*K-RAS & APC*	NEG	ADENOMA	
5	*TP53*	POS	B	T3N0MX
13	*K-RAS*	POS	B	T3N0MX
20	*K-RAS*	NEG	C1	T2N2MX
21	*BRAF*	POS	C1	T3N2MX
23	*K-RAS*	POS	RECURRENCE	
26	*K-RAS*	NEG	B	T4N0MX
27	*BRAF*	POS	D	T4N2M1
28	*TP53*	POS	B	T3N0MX
29	*K-RAS*	POS	B	T3N0MX
31	*K-RAS*	POS	B	T4N0MX
32	*K-RAS*	POS	B	T3N0MX
33	*BRAF*	POS	C1	T3N1MX
35	*BRAF*	POS	C1	T3N1MX
36 & 37	*K-RAS*	POS	D	T3N1M1
42	*K-RAS*	POS	D	T3N0M1
45	*APC*	NEG	C1	T3N2MX
47	*APC*	NEG	B	T3N0MX
48	*BRAF*	POS	C2	T4N2MX
49	*K-RAS*	POS	A	T1N0MX

Table 6.4 Summary of results of peritoneal washings and post-operative drain fluids Details of the mutation detected, whether any peritoneal sample gave a positive result and the stage of the tumour are given.

Of the patients with positive results, one was already suffering from intra-abdominal recurrence, having undergone right hemicolectomy for a Dukes' B tumour 14 months previously. Three other patients (including one with two tumours bearing *K-ras* mutations) had hepatic metastases and were thus Dukes' stage D. Four patients had Dukes' C tumours, seven Dukes' B and one Dukes' A.

The patients with Dukes' B tumours are an interesting group to study, because they would not normally be considered for adjuvant chemotherapy. Of these patients, one patient had a T4 tumour (number 31), with abscess formation, which was adherent to the vagina. Tumour was present at the resection margin, and therefore this patient is at high risk of locally recurrent disease in the pelvis. Two other patients (tumours 2 and 13) had

tumours that clinically involved a loop of small bowel. In neither case, however, was the pathological stage given as T4. One of these patients presented with small bowel obstruction 10 months post-operatively, and at operation was found to have recurrent disease in the pelvis, which was confirmed histologically. She sadly died of complications after this second operation. A further patient (tumour 32) had an advanced tumour at presentation, and had undergone defunctioning colostomy and pre-operative radiotherapy. It is therefore possible that this patient's tumour had been of a more advanced stage before treatment began. The accurate identification of lymph nodes after radiotherapy can be particularly difficult. A fifth patient (tumour 5) had synchronous bulky tumours, and the surgeon was uncertain as to whether he had been able to perform a curative procedure. The remaining 2 patients with Dukes' B tumours and the patient with a Dukes' A tumour had no obvious clinical or pathological risk factors for recurrent disease.

Of the patients without positive peritoneal samples, one had a large adenoma, requiring anterior resection and two others had Dukes' B tumours (although one of these (26) was a T4 lesion). Of the two Dukes' C tumours, one was a T2 tumour, and the other T3. Both were found to have vascular invasion on histological examination, and may perhaps be more likely to recur at distant sites rather than locally.

CHAPTER 7: IS MACS NECESSARY?

7.1 Introduction

PCR based techniques rely on the amplification of DNA, which may initially be present in only tiny amounts. It makes sense intuitively that if we are looking to detect rare DNA, such as a colorectal cancer cell containing a specific mutation, against a background of large amounts of other DNA not containing the mutation, that if we can increase the ratio of abnormal to normal DNA this will improve our chances. We therefore began our project by using MACS separation on every sample. This technique has been shown in our laboratory to be useful in the detection of malignant cells in the peritoneal cavity (Wong *et al.*, 1996). MACS has also been used by other workers looking for small numbers of malignant cells, for example tumour cells in the mesenteric circulation (Iinuma *et al.*, 2000). The principle of the MACS technique is described in section 2.3.2.1.

We have demonstrated that only about 1% of cells are in the positive fraction of our samples after MACS separation (section 4.3). If most of the malignant cells in the samples are retained in the positive fraction then this procedure gives a one hundred fold enrichment of the target cells for our detection step, and should increase our ability to detect rare malignant cells if they are present. The MACS process also allows for the removal of any red blood cells that may remain despite density centrifugation. Haemoglobin from these cells, which may be incompletely eliminated during DNA extraction, could reduce the performance of PCR in later steps.

However, there are some theoretical and practical disadvantages to the routine use of MACS. The cell counts of the whole cell pellet after density centrifugation, compared with the total number of cells in the positive and negative fraction added together, show that on average about 30% of cells are lost during the repeated manipulations required to label the cells with magnetic microbeads and separate them in a miniMACS column (see section 4.4). If only small numbers of malignant cells were present in the samples before

separation, and particularly if cancer cells were more vulnerable to being damaged or destroyed in the process, then MACS may be counter-productive, especially in samples with low initial numbers of cells.

On a practical level, it is important that if our methods do allow us to detect a group of patients who may benefit from extra treatment or more intensive surveillance, that the techniques are widely applicable. It would therefore be advantageous if our methods could be streamlined as far as possible. PCR and MLA techniques can be performed on DNA from batches of samples, which can be stored in a freezer until a convenient time. MACS needs to be performed on each sample or group of samples immediately after density centrifugation, on the day of specimen collection. Even leaving the samples on ice overnight appeared to lead to unacceptable numbers of non-viable cells when counted using trypan blue, although this was not quantified. The combination of density centrifugation, and MACS with the combination of antibodies described, took approximately three and a half hours to perform depending on the number of samples collected. This could be inconvenient, especially if surgery took place on an afternoon operating list and samples were not brought to the lab until early evening.

For these reasons we decided to investigate whether the MACS step improved our ability to detect malignant cells in the peritoneal cavity, or whether it could possibly be impairing our ability to detect such cells. Attempts to answer this question using spiking experiments produced variable results (see section 4.8.2). We therefore addressed the problem further with our clinical samples.

Peritoneal samples from the last 17 patients to be recruited (19 tumours, 50 peritoneal samples) were split after density centrifugation, with half of the sample being pelleted and frozen without further treatment and half the sample being separated using the usual MACS process. When a mutation was detected in one of the 19 primary tumours then DNA was extracted from the non-separated cell pellets and from the positive fractions of the relevant peritoneal samples and both were analysed by the appropriate technique (MLA or mutation specific PCR).

7.2 Results

Mutations were detected in DNA from 11 of the 19 primary tumour samples. Tumours 36 and 37, from the same patient, are considered together for the remainder of this chapter. Five patients had tumours with K-ras mutations (tumour numbers 31, 32, 36/37, 42 and 49), two had APC codon 1309 mutations (tumour numbers 45 and 47) and three had BRAF mutations (numbers 33, 35 and 48). Each peritoneal sample, both separated and non-separated, was tested at least twice by the appropriate method, using products from independent PCR reactions in the case of MLA, before being dismissed as negative.

	MACS separated samples				samples not separated			
tumour	preop	postop	day1	day2	preop	postop	day1	day2
31		+	+		+	+	-	
32	+	+	+	+	+	-	-	-
33	+	+			+	+		
35	+	-	+		+	-	-	
36/37	+	+			-	+		
42	+	+	-		-	-	-	
45	-	-	-		-	-	-	-
47		-	-	-	-	-	-	
48	+	-	-		-	-	-	
49	+	+			-	-		

Table 7.1 showing comparison of results from separated and non-separated samples. + Indicates a positive result, - indicates a negative result. An empty cell indicates that no sample was available. Pre-op= wash at start of operation, post-op= wash at end of operation, day 1 = drain fluid from first post-operative day, day 2 = drain fluid from second post-operative day.

Table 7.1 shows a comparison of the results obtained from the positive fraction after MACS separation with the results from the non-separated samples. 26 pairs of samples were available for comparison. In four cases only a single sample was available; one

sample was not subjected to MACS separation, and three samples did not yield amplifiable DNA (two after MACS, one not separated).

Considering only those samples for which paired results are available, a positive result was found significantly more often in samples subjected to MACS separation, than in those not separated. These results are shown in table 7.2.

	Separated	Non-separated
Positive	17	6
Negative	9	20
	26	26

Table 7.2 comparing proportion of positive results in samples with and without MACS separation. P = 0. 0047 Fisher's exact test

It is interesting to compare the results from the separated and non-separated halves of each sample. Of the twenty-six pairs of samples, the results of the separated and non-separated halves were the same in 15 cases. Both were positive in 6 pairs, both were negative in 9 pairs. For a further 11 pairs of samples a positive result was seen in the samples after MACS, with a negative result (sometimes a questionable positive seen on the gel image) in the non-separated sample. There was no case in which a positive result was seen in the non-separated sample but which gave a negative result with the positive fraction after MACS. This shows that MACS is not impairing our ability to detect cells with the relevant mutations when these are present.

If we consider that any positive result for a particular patient may put them into a higher risk group for recurrence, then it is important to consider these results in terms of patients rather than numbers of samples. Of the 10 patients, 5 had at least one positive peritoneal sample with both techniques and 2 had no positive results with either. The remaining 3 patients were 'upgraded' from having negative to positive peritoneal samples by the use of MACS separation before molecular analysis.

It seems likely that any benefit gained from MACS separation would be greatest in samples with largest numbers of cells in the starting material, where, for the same number of malignant cells in a sample, the ratio of abnormal to normal cells would be the least. However, comparing the mean number of cells in the samples giving positive results both with and without separation, and those giving positive results only after MACS, no difference was noted (mean number of cells in initial samples 2.7×10^7 vs. 3.2×10^7 p=0.5).

An example of a gel showing positive results in separated, but not whole samples is shown below (Figure 7.1).

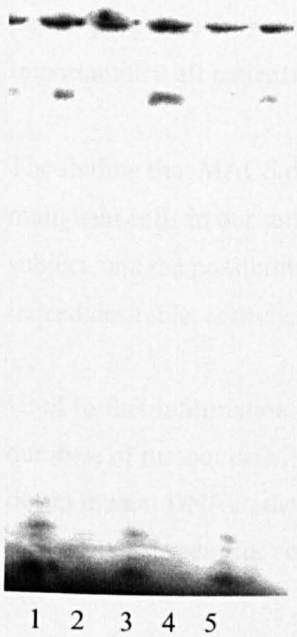


Figure 7.1 Gel showing mutant K-ras in separated, but not non-separated, peritoneal samples. Lane 1 tumour 42, lanes 2 and 3 pre-operative wash, whole sample and separated respectively, lanes 4 and 5 post-operative wash, non-separated sample and separated sample. Positive results seen in tumour sample and both separated samples (Lanes 1, 3 & 5).

7.3 Discussion

Despite studying a relatively small number of samples we have shown that mutant DNA is significantly more likely to be detected in peritoneal washings or post-operative drain fluids if efforts are first made to enrich any epithelial cells present. This confirms our initial assumption that the use of MACS would be beneficial for the detection of rare mutant cells.

In our group of ten patients, three were reclassified as having positive peritoneal samples when comparing results after MACS with those using non-separated samples. Although numbers are too small to test for statistical significance, if longer follow-up reveals a poorer prognosis for patients with mutant DNA in the peritoneal cavity, then it is important that all patients are classified as accurately as possible.

The finding that MACS does seem to have an important role in enabling the detection of malignant cells in our samples may limit the general applicability of our techniques. This subject, and the possibility that our techniques may be more sensitive than is necessary or indeed desirable, is discussed further in chapter 8.

Until further information about what sensitivity is appropriate in the detection of small numbers of tumour cells, we have demonstrated that MACS does improve our ability to detect mutant DNA in the peritoneal washings and post-operative drain fluids of patients undergoing surgery for colorectal cancer.

CHAPTER 8 : GENERAL DISCUSSION

Local recurrence is a common problem in colorectal cancer, and until recently, the perception has been that it is inevitable in certain groups of patients, and almost uniformly fatal. Recent advances in surgical technique and the growing use of adjuvant treatment have helped to reduce the incidence of local recurrence. Evidence is also emerging that close follow up, using appropriate combinations of established techniques (CT scanning and CEA measurement) and newer techniques such as PET scanning, may allow detection of patients with local recurrence at a stage when salvage treatment is possible. Our aim was to describe techniques, which would allow isolated tumour cells, the likely precursors of local recurrence, to be detected in the peritoneal cavity around the time of surgery. If patients in whom such cells are detected do prove to have a poorer outcome, then they may be suitable candidates for adjuvant chemotherapy or targeted post-operative surveillance.

Those patients with spread to the lymph nodes (Dukes' stage C or pN1 or 2) normally receive adjuvant treatment under current protocols. Our initial aim, therefore, in attempting to detect colorectal cancer cells in the peritoneal cavity was to select a group of patients with favourable histology (who would normally be treated by surgery alone) who we felt were at high risk of local recurrence. At first glance we have been successful in this, with eight of the sixteen patients in our positive peritoneal group having Dukes' A or B tumours. However, as we have seen, closer inspection of the clinical and histological findings in five of these patients, could lead to their classification as being at high risk of recurrence. This perhaps emphasises the inadequacy of any single staging system taken in isolation, and shows the importance of good provision of information from surgeon to pathologist and vice versa. However, the detection of isolated tumour cells in the peritoneal cavity could be useful in highlighting the increased risks faced by these patients.

Of the 16 patients with mutant DNA detected in the peritoneal cavity at some stage during or after surgery, 10 are alive and well. Four patients are alive with evidence of

cancer; one of these was undergoing surgery for intraperitoneal recurrence when the samples were collected, a second has recurrent malignant melanoma and the other two have liver metastases. Two patients have died; one death followed surgery for local recurrence (10 months after the index operation) and the other was from pelvic recurrence (the patient having had a T4 tumour with liver metastases at presentation). Of the five patients with a mutation detected in their primary tumours, but without mutant DNA in the peritoneal samples, one died in the early post-operative period (of a myocardial infarction), and the other four are alive and well. These follow-up data are not of sufficient duration to allow us to assess whether the patients with malignant cells in their peritoneal cavities are at increased risk of local recurrence. Problems may continue to manifest themselves for a number of years after primary surgery, and we will need to review the patients' notes again in the future. A review of the notes in September 2007 would allow a minimum of five-year follow up for all patients, although three years would probably be adequate to allow the vast majority of recurrences to be seen.

One of our secondary aims was to quantify any malignant cells we detected. We felt that, although RT-PCR has a number of disadvantages, previously outlined in section 1.5.4.2, it would be suitable for use in those samples in which we had previously found mutant DNA by MLA or mutant-allele specific PCR. RT-PCR can be performed in a quantitative fashion, and an epithelial-specific marker, such as cytokeratin 20 would have been a suitable target. Indeed, a commercially available kit allows such tests to be performed. Unfortunately, it did not prove possible to extract RNA of sufficient quality from our frozen cell pellets, without sacrificing the DNA necessary for our diagnostic tests. Attempts to extract both nucleic acids from the same sample produced poor quality DNA and minimal RNA, which was inadequate for further use. We therefore abandoned our aim to quantify cells in our samples. This is unfortunate, because, as discussed below, the appropriate level of sensitivity for molecular biological tests for minimal residual disease is not known. We were surprised to detect isolated tumour cells in samples from so many of our patients, and it may be that these cells are so small in number that they are unlikely to cause recurrent disease. It is also uncertain whether these cells are viable (i.e. capable

of implantation and replication), although we do know that they are capable of excluding a vital dye.

Our final aim was to streamline our tests, so that they would be simple to apply and therefore more widely applicable. One major barrier to this has been the finding, described in the previous chapter, that the use of MACS separation on the samples increases the likelihood that malignant cells will be found in peritoneal washing and drain fluid samples. Although we did not combine the PCR or MLA reactions for the various mutations studied, these can each be performed on batches of samples, which can be stored at -20°C until needed. The MACS process, however, is moderately time consuming, and often needs to be performed at inconvenient times, with relatively small numbers of samples in each batch. These problems arise from the observation that leaving the samples even overnight (at 4°C) leads to a high proportion of cells unable to exclude trypan blue. MACS is also costly with each miniMACS column costing almost £6. The column is only intended to be used for a single sample and in practice cannot be used for more than two samples. The antibodies required also add to the expense of the procedure. This combination of factors means that the combination of techniques we have described is more likely to remain a research tool, rather than being routinely applicable in clinical laboratories.

To overcome these problems, several approaches could be used. It may be possible to simplify the MACS technique used. For example, the use of a single stage reaction for binding microbeads to epithelial cells would save time and possibly money. A human epithelial antibody (HEA), directly conjugated to magnetic microbeads, has become available and may be suitable for this purpose.

Testing up to four samples per patient, taken from the peritoneal cavity at different times, also increases the volume of work. Follow up of our patients may indicate that it is only involvement of the peritoneal washings **or** the drain fluids that is significant, or perhaps even just one of these samples. If the number of samples per patient could be reduced from a maximum of four to just one or two, either by collecting fewer samples or by

pooling samples together for analysis, then savings in time and equipment could be considerable. It is interesting to note that only one patient who had any positive washing or drain fluid had a negative result in the first washing sample. However, the continued finding of malignant cells in the peritoneum after removal of the primary tumour may be of greater significance in determining patients' outcomes. Cells present at the time of surgery could be removed with the tumour or by peritoneal lavage; cells in the drain fluid may have been shed from cut lymphatic channels and, because of the time when they are released, cannot be removed by conventional treatment. The role of intraperitoneal chemotherapy in the early post-operative period may be relevant and is under study (de Bree *et al.*, 2002). Administering chemotherapy directly into the peritoneal cavity of patients at high risk of local recurrence allows a greater concentration of drug to be applied to the tumour cells, without increasing systemic toxicity. This is advantageous given the relative chemo-resistance of colorectal tumours.

One other possible alternative to the use of MACS, would be to increase the sensitivity of the reaction to detect mutant DNA, whether this is a MLA or a mutation specific PCR. If the sensitivity was improved, without sacrificing specificity, then it might be possible to abandon the enrichment step using MACS.

One major advantage of using MACS is that it allows us to overcome a common criticism of PCR based techniques for the detection of minimal residual disease. Mutant DNA in peritoneal washings or drain fluids could be present in a free state after cell lysis, or the cells containing the mutant DNA could be lethally damaged, and therefore have no significance for the prognosis of the patient. The repeated washing and centrifugation steps during MACS ensures that free DNA will be washed away, and is likely to result in the loss of damaged cells.

As discussed in chapter 5, using the combination of techniques described we were able to detect one or more mutations in almost half of our panel of colorectal tumours (22 out of 46 tumours). This is a reasonable result given the wide variety of different genes mutated in colorectal cancer and the huge number of possible carcinogenic mutations in those

genes. What could be done to increase the proportion of tumours in which we can detect a mutation? It would be possible to look for the other *TP53* mutations for which reaction conditions are described by Dong (2001). We initially decided not to do this because of the lack of a readily available positive control for these reactions. Given some of the difficulties in interpreting the p53 results, an appropriate positive control is particularly important. Although the commonest position for a *K-ras* mutation is codon 12B, mutations are also described at codons 12A and 13. Jen (1994*b*) described reaction conditions for the detection of these mutations. Again, other cell lines would need to be searched to find one with a suitable mutation to act as a positive control. An alternative would be to test a number of tumours, and if one gave positive results, with a mutation confirmed by sequencing, this could be used as a control in further experiments. It may also be possible to use deliberately mutated DNA for this purpose. It would probably be possible to design an MLA reaction to look for the point mutation at codon 1450 of APC, if a shorter PCR product was used.

If we continue to detect further mutations in the same small number of genes, which are mutated in colorectal tumours developing via the same pathway, it is likely that a significant proportion of the mutations we detect will be second or third mutations in a particular tumour. While this may provide interesting information, it would be more helpful to identify a first mutation in one of the tumours whose peritoneal samples we are currently unable to study.

All the mutations we have sought to detect occur most commonly in tumours developing by the classical pathway described by Fearon and Vogelstein (1990), so-called microsatellite stable or loss of heterozygosity tumours. As described in chapter one, about 15% of sporadic colorectal cancers develop via a different pathway, which is similar to those tumours seen in patients with HNPCC, via abnormalities in DNA mismatch repair, so-called microsatellite instability (MSI) or replication error positive tumours. Because they have a different mutation spectrum when compared with microsatellite stable tumours, the techniques we have described are less likely to detect MSI tumours. For example, *TP53* mutations are rare in MSI tumours. To increase the prospect of detecting

such tumours, it may be possible to design tests to detect mutant forms of one of the genes commonly mutated in microsatellite unstable tumours, such as those for the TGFβ receptor or hTCF4 (see section 1.6.3.3). If it were possible to design sufficiently sensitive tests, it would be preferable to attempt to detect further mutations with allele-specific PCR rather than MLA. Simple PCR reactions are less time-consuming and avoid the inconvenience and risks associated with the use of ^{32}P. The use of such tests could perhaps increase the proportion of tumours in which we are able to detect a mutation from about half to almost two thirds.

Microsatellite instability itself is detected by PCR reactions designed to amplify a number of specific sites, where increases or decreases in length of a particular microsatellite have been well characterised. DNA from tumours with MSI will show alteration in product length, which appears as a ladder pattern or as a new band adjacent to the expected band (Ward *et al.*, 2001). A number of groups have described tests for DNA with microsatellite instability against a background of normal DNA in faeces (Ahlquist *et al.*, 2000; Rengucci *et al.*, 2001). It seems likely that such tests could be modified to study peritoneal washings and drain fluids.

It was reassuring that we were able to confirm most of the mutations (16 of 23) detected in our primary tumour samples by sequence analysis. This, in combination with the rigorous use of controls in all experiments, means that false positive results for mutations in tumours are unlikely. The tumours in which the mutation could not be confirmed by sequencing may be those where there were, for example, a large number of inflammatory cells (leucocytes, with wild type DNA) within the tumour, diluting the mutant DNA. Ongoing work in our laboratory is attempting to provide additional evidence about the validity of some of our results, particularly the detection of *K-ras* mutations. A series of mutation-specific PCR amplifications for the various *K-ras* codon 12 mutations has been developed, and the samples described in this thesis are soon to be analysed. It will be interesting to compare the specificity and accuracy of the two techniques.

Malignant cells in the peritoneum have previously been detected in about one quarter to one third of colorectal cancer patients, using the various techniques described in chapter one. Although we hypothesised that the use of molecular techniques would increase this proportion, we were somewhat surprised to find such cells in 75% of the patients in whom we could detect a mutation in the primary tumour. One explanation for this is that tumours with the mutations we were able to detect have a particularly poor prognosis, and that they therefore have a higher rate of peritoneal involvement than our population of tumours as a whole. However, there was no difference in the proportion of primary cancers in Dukes' stage A and B compared with C and D between those in which a mutation was detected, and those in which no mutation was found. The largest group of mutations we detected were of *K-ras* codon 12B. Mutations in the *ras* gene have been associated with a poorer prognosis, but the relationship, as with many molecular predictors of disease behaviour, is complex, and the increased risk may apply only to certain mutations, particularly G→T, which was rare in our series (Houlston, 2001).

One other explanation for the high frequency of detection of tumour cells in these samples is that our techniques are too sensitive, meaning that large numbers of patients may be classified as being at increased risk of recurrent disease when this is not appropriate. For example, the ability to detect one tumour cell in 10^7 normal cells, which is claimed for some molecular biological techniques, may allow the detection of DNA from tiny numbers of cells that are not destined to cause problems for the patient. The processes required for an isolated tumour cell to establish itself as a peritoneal metastasis are complex, and it is likely that most free tumour cells die: experiments with transplantable tumours in mice indicate that very large numbers of cells may need to be injected in order to establish a tumour. The clinically significant detection threshold for malignant cells in any potential site of metastasis (such as bone marrow, lymph nodes or peritoneum) has not been calculated, and may need to be specified for each technique in use. Our MLA and mutant-allele specific PCR tests were able to identify one tumour cell in about one thousand normal cells. When combined with an enrichment of perhaps one hundred fold by the use of MACS, this gives the ability, under optimum conditions, to detect one tumour cell in 10^4 to 10^5 normal cells. No data are available about whether

tumour cells present in the peritoneal cavity at this dilution are able to establish themselves as metastatic lesions. An added complication with our samples is the extreme heterogeneity of the starting material, with the number of cells varying by a factor of 10^3. The lack of a technique to determine the proportion of malignant cells in the sample, as might have been possible with RT-PCR, makes interpretation of the results more difficult. If the combination of techniques we have used does prove to be too sensitive, with a low risk of local recurrence in the patients in whom we have detected isolated tumour cells, it may be possible to omit the MACS step without compromising the accuracy of our assessment. The results from those patients where both separated and non-separated samples are available (described in chapter 7) may be helpful in determining this. If MACS proved unnecessary this would, as described above, increase the applicability of our techniques, by removing the least 'streamlined' part of the process.

Whether further, similar work will prove useful in making decisions about patient management is not yet clear. We will continue to follow the progress of all the patients who kindly took part in the study. If future work were considered, it would probably be reasonable to collect only a peritoneal washing at the start of operation, as this was positive in all but one of the patients who had any positive result. The intended confirmation of our *K-ras* results using MASA will add weight to our results, and may allow the use of a non-radioactive technique for further work, which would obviously be advantageous. The technique described for the detection of *BRAF* mutations has not been used previously, and could be applied to other situations where DNA based techniques have been used for the detection of minimal residual disease.

APPENDIX

A.1 MACS Protocol

1. After density centrifugation cells were resuspended in 1ml of ice cold PBS, and centrifuged at 2500rpm for 4 minutes.
2. The pellet was resuspended in 20µl of mouse anti-human Ber-EP4 antibody. If the cell pellet was too large to resuspend in this volume, 80µl of cold PBS was added. This mixture was incubated for 30 minutes on ice.
3. The sample was washed in 1ml of cold PBS as before.
4. The pellet was resuspended in 20µl of secondary antibody, goat anti-mouse, conjugated to magnetic microbeads, with 80µl of cold PBS, and incubated on ice for 20 minutes.
5. The sample was washed as before.
6. The sample was resuspended in 0.5ml of ice cold MACS buffer (PBS containing 0.5% bovine serum albumin (Sigma-Aldrich) and 2mM EDTA (Gibco BRL))
7. A miniMACS column (Miltenyi Biotec) was positioned in the specially designed magnet (Miltenyi Biotec), and prepared by allowing 0.5ml of MACS buffer to run through (this was discarded).
8. A labelled 2ml eppendorf was positioned under the column, and the sample introduced into the top of the column. As it ran through the negative fraction was collected. When the top of the column was nearly empty, 0.5ml of MACS buffer was added, and this was repeated twice more, giving a total volume of 2ml for the negative fraction.
9. The column was removed from the magnet, and put into a second labelled eppendorf. 1ml of MACS buffer was introduced into the column and was forcibly pushed through with the 'plunger' provided with the column. This was repeated once, giving a total volume of 2ml for the positive fraction.
10. 50µl from each sample was removed and mixed with trypan blue dye for cell counting.

11. The remaining fractions were centrifuged at 3000rpm for 8 minutes, the supernatants were discarded and the cell pellets frozen at –80°C until needed for further experiments.

A.2 PCR

A.2.1 Primer sequences

All sequences are given 5' to 3'; primers were obtained from Invitrogen.

K-ras exon 1

Sense: AGG AAT TCA TGA CTG AAT ATA AAC TTG

Anti-sense: ATC GAA TTC CTC TAT TGT TGG ATC ATA TTC

K-ras used for sequencing

Sense: TTA AGC GTC GAT GGA GGA GT

Anti-sense: GAA TGG TCC TGC ACC AGT AA

P53 exon 8

Sense: AGT GGT AAT CTA CTG GGA CG

Anti-sense: TGA GGC TCC CCT TTC TTG C

APC wild type

Sense: GAC GAC ACA GGA AGC AGA TT

Anti-sense: GCT TGC TTA GGT CCA CTC TC

APC 1309 mutation, allele specific primer

Sense: TAG CAG AAA TAA AAG ATT GG

Anti-sense: as anti-sense for APC wild-type

BRAF exon 15, for MLA (43 bp product)

Sense: ACC CAC TCC ATC GAG ATT TC

Anti-sense: GGT GAT TTT GGT CTA GCT AC

BRAF exon 15, for sequencing (224 bp product)

Sense: TCA TAA TGC TTG CTC TGA TAG GA

Anti-sense: GGC CAA AAA TTT AAT CAG TGG A

A.2.2 PCR reaction conditions

All programmes were linked to a long hold at 4°C

K-ras exon 1

94°C x 5 minutes

39 cycles of: 94°C x 45 seconds

58°C x 45 seconds

72°C x 45 seconds

72°C x 5 minutes

K-ras sequencing primers

94°C x 5 minutes

39 cycles of: 94°C x 30 seconds

52°C x 30 seconds

72°C x 45 seconds

72°C x 5 minutes

p53 exon 8, wild type APC, BRAF Sequencing primers

95°C x 5 minutes

35 cycles of: 95°C x 30 seconds

62°C x 30 seconds

72°C x 30 seconds

70°C x 5 minutes

APC 1309 mutant

94°C x 5 minutes

39 cycles of: 94°C x 45 seconds

59°C x 45 seconds (stringent conditions)

(OR 57°C x 45 seconds for less stringent conditions)

72°C x 45 seconds

72°C x 5 minutes

BRAF primers for MLA

95°C x 5 minutes

35 cycles of: 95°C x 30 seconds

55°C x 30 seconds

72°C x 30 seconds

72°C x 5 minutes

A.2.3 PCR reaction mixtures

Most reagent concentrations remained the same for all PCR reactions and are listed in table A.1. Each PCR was optimised with respect to the magnesium concentration, see table A.2. The change of Taq polymerase supplier from Invitrogen to MBI Fermentas required some reactions to be re-optimised, and certain reactions were only successful (or attempted) with one or other enzyme, as shown in table A.2.

Reagent	Volume µl
10 x PCR buffer (without magnesium) as supplied with Taq	2.5
Magnesium chloride, as supplied with Taq	See table A.2
dNTP (10mM each) (Gibco BRL)	0.5
Sense primer (100ng/µl) (Invitrogen)	0.25
Anti-sense primer (100ng/µl) (Invitrogen)	0.25
Taq polymerase (5 units/µl) (See table A.2 for supplier)	0.125
Template DNA	2
Sterile water	To 25µl

Table A.1 showing reaction mixture for a 25µl PCR reaction.

PCR reaction	Invitrogen Taq	Fermentas Taq	Magnesium concentration (mM)	Volume of 50mM $MgCl_2$ (µl)	Volume of 25mM $MgCl_2$ (µl)
K-ras	Yes		3	1.5	
K-ras		Yes	4		4
K-ras sequencing		Yes	1.5		1.5
TP53	Yes	Yes	3	1.5	3
APC 1309, allele specific. Stringent conds.	Yes	No	2	1	
APC 1309, allele specific. Sensitive conds.		Yes	2.5		2.5
APC wild type	Yes	Yes	3	1.5	3
BRAF for MLA	No	Yes	4		4
BRAF for sequencing		Yes	2		2

Table A.2 showing magnesium concentration, volume of magnesium chloride and supplier of Taq polymerase for each PCR reaction.

A.3 Mismatch Ligation Assay

A.3.1 Mismatch Ligation Assay (MLA) Oligonucleotides

All oligonucleotides were obtained from Invitrogen

Oligonucleotides for *K-ras*

12B common (end-labelled): TGG CGT AGG

12B ALA (mutation specific): TGG AGC TGC

12B ASP (mutation specific): TTG GAG CTG A

12B VAL (mutation specific): GTT GGA GCT GT

12 BLOCKER: AGC TGG TGG CG

Oligonucleotides for *TP53*

P53/273CT3 (end-labelled): GTG TTT GTG

P53/273GA3 (end-labelled): TGT TTG TGC C

P53/273CT5 (mutation specific): TTG AGG TGT

P53/273GA5 (mutation specific): TTG AGG TGC A

P53 BLOCKER: AGG TGC GTG TT

Oligonucleotides for *BRAF*

BRAF 3 (end-labelled): GAA ATC TCG A

BRAF 5 (mutation specific): TAG CTA CAG A

BRAF BLOCKER: ACA GTG AAA TC

MLA reaction	End-labelled oligo	Mutation specific oligo	Blocking oligo
K-ras	2	2	100
TP53	5	25	100
BRAF	2	25	200

Table A.3 showing concentration of oligonucleotides ('oligo') used in each MLA reaction. All concentrations in ng/µl.

A.3.2 Protocol for Mismatch Ligation Assay

1. Reagents for end-labelling reaction were combined. For 20 samples:
    - Oligonucleotide for end labelling, 22µl (for p53 experiments, 11µl of each oligonucleotide)
    - T4 polynucleotide kinase 10U/µl, 2.2µl
    - T4 polynucleotide kinase buffer, 4.4µl 10 x buffer A for Fermentas T4 kinase or 8.8µl 5 x forward reaction buffer for Invitrogen T4 kinase
    - Sterile water to 42µl
    - ^{32}PγATP 40µCi, 2µl
2. The mixture was heated to 37°C for a minimum of 10 minutes (Invitrogen T4 kinase) or 30 minutes (Fermentas T4 kinase). All heating steps were performed on a Hybaid thermal cycler.
3. T4 kinase was heat inactivated by heating to 65°C for 10 minutes.
4. Reagents for master mix were combined. For 20 samples:
    - Each mutation specific oligonucleotide, 22µl
    - Blocking oligonucleotide, 22µl
    - Spermidine 20mM, 22µl
    - T4 gene 32 protein, 5µg/µl, 12µl
    - T4 ligase buffer, 88µl of 5 x T4 ligase buffer for Invitrogen T4 ligase or 44µl of 10 x T4 ligase buffer for Fermentas T4 ligase.
    - Saline to 220µl
5. 10µl of master mix was placed in each labelled 0.5ml eppendorf, and 8µl of appropriate PCR product added.
6. 2µl of end labelled oligonucleotide mixture was added to each tube.
7. Tubes were heated to 95°C for 5 minutes to denature DNA, and allowed to cool to room temperature for 15 minutes while annealing occurred.
8. 1 unit of T4 ligase was added to each tube. For Invitrogen T4 ligase this was 1µl of 1 unit/µl enzyme, for Fermentas T4 ligase the 5 units/µl enzyme was first diluted 1 in 5 with T4 ligase buffer.

9. Tubes were incubated for 1 hour at 37°C.
10. T4 ligase was heat inactivated by heating to 68°C for 10 minutes.
11. 1µl of alkaline phosphatase (1U/µl), mixed with 2.4 µl of dephosphorylation buffer provided with the enzyme (Roche) was added to each tube, and incubated at 37°C for 30 minutes.
12. 5µl of denaturing loading dye was added to each tube, and samples heated to 90°C for 5 minutes to denature DNA. Each 5ml of loading dye contained
    - 4.3ml formamide
    - 0.1ml 1.25% bromophenol blue
    - 0.1ml 1.25% xylene cyanol
    - 0.5ml 0.5M EDTA pH 8
13. Samples were loaded onto a pre-warmed 12% denaturing polyacrylamide gel. Each 50ml gel mixture contained
    - 21g urea (BDH, Poole, UK)
    - 5ml 5 x TBE
    - 29.45ml sterile water
    - 15ml 40% w/v solution of acrylamide (38%) and bis-acrylamide (2%), 19:1 ratio in deionised water (Amresco, Ohio USA)
    - 50µl TEMED (N,N,N',N'-Tetramethylethylenediamine, Sigma-Aldrich, Steinheim, Germany)
    - 500µl 10% ammonium peroxodisulphate (BDH, Poole, UK)
14. The gel was subjected to electrophoresis at 300V, with 0.5 x TBE as running buffer, using a V15-17 vertical gel electrophoresis apparatus (Gibco BRL). Electrophoresis continued for approximately one hour 45 minutes, until the dye front reached the bottom of the gel.
15. The gel was removed from the gel tank, and the smaller glass plate removed. The gel was immersed in a solution of 10% v/v acetic acid and 20% v/v methanol in deionised water for 10 minutes.
16. The acetic acid/methanol was poured off and the gel immersed in 20% v/v glycerol in deionised water for 30 minutes.

17. The glycerol was poured off, the gel dried with tissue paper and transferred to a double thickness of 3mm Chr Whatman chromatography paper. The gel was covered with cling film.
18. The gel was transferred to a gel drier (Hoeffer Scientific Instruments, San Francisco, USA) and heated to 80°C for 3 hours, with a vacuum of –30 inHg applied overnight.
19. The gel was wrapped completely in cling film and placed in a phosphoimager cassette, which had previously been blanked.
20. The gel was exposed for four hours and then read on a phosphoimager (Fuji). The gel image was saved in .img format.

A.4 Gel Pictures for Tumours with Mutations

A.4.1 Additional gel pictures for tumours positive for *K-ras* mutations

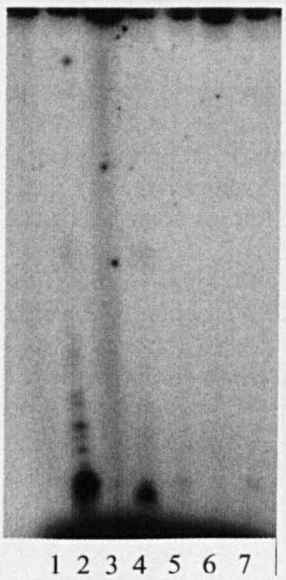


Figure A.1
MLA Kras experiment 1.
Lane 1 water
Lane 2 positive control
Lane 3 tumour 9
Lane 4 tumour 13
Lane 5 normal DNA
Lanes 6 & 7 tumours 12 & 1

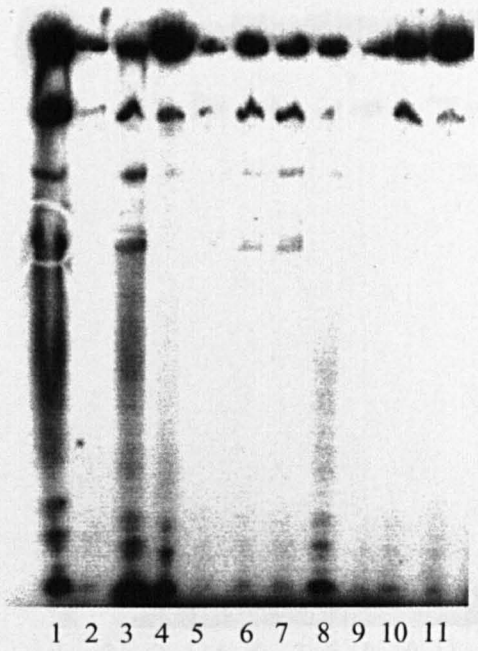


Figure A.2
K-ras experiment 29, showing positive results in lanes 1, 3, 4 & 8.
Lane 1 positive control
Lane 2 negative control
Lane 3 tumour 2
Lane 4 day 1 drain fluid from patient with tumour 2
Lanes 5-7 tumours 14, 32 & 36
Lane 8 tumour 37
Lanes 9-11 drain fluids from patients without cancer. Negative control for inflammatory cells in peritoneum

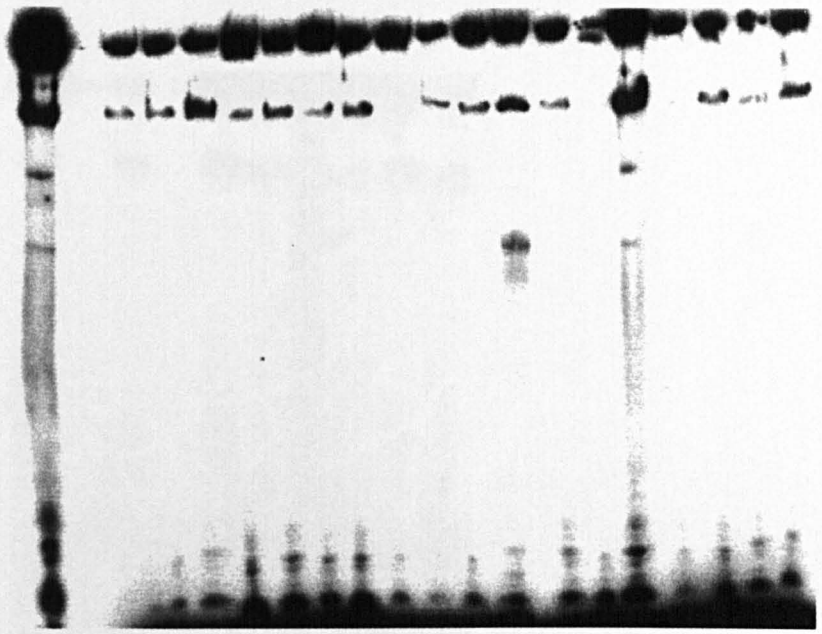


Figure A.3
K-ras experiment 23, showing positive results in lanes 1, 5-10, 12-16, 18-20.
Lane 1 positive control
Lane 2 empty
Lane 3 negative control
Lane 4 tumour 32
Lane 5 tumour 36
Lane 6 tumour 29
Lanes 7 & 8 peritoneal samples from patient with tumour 29
Lane 9 tumour 49
Lanes 10-12 peritoneal samples from patient with tumour 49
Lane 13 tumour 31
Lanes 14-18 peritoneal samples from patient with tumour 31
Lane 19 tumour 32
Lane 20 tumour 36

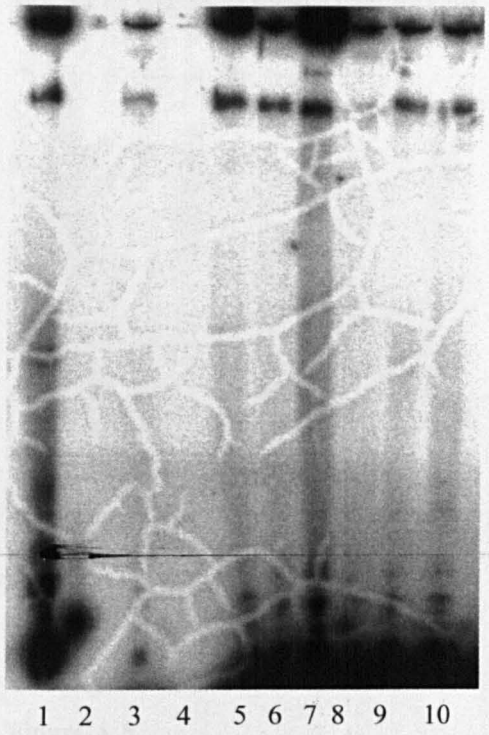


Figure A.4
K-ras experiment 24 showing clearly positive results in lanes 1, 7 & 10. Possible positive results in lanes 5, 6, 8 & 9 are more difficult to interpret because of gel cracking.
Lane 1 positive control
Lanes 2 & 4 empty
Lane 3 negative control
Lane 5 tumour 31
Lanes 6-9 peritoneal samples from patient with tumour 31
Lane 10 tumour 42

1 2 3 4 5 6 7 8 9 10 11

Figure A.5
K-ras experiment 16 showing positive results in lanes 2, 5, 8 & 11.
Lane 1 negative control
Lane 2 positive control
Lanes 3 & 4 tumours 21 & 22
Lane 5 tumour 23
Lanes 6 & 7 tumours 24 & 25
Lane 8 tumour 26
Lanes 9 & 10 tumours 27 & 28
Lane 11 tumour 29

A.4.2 Additional gel picture for tumour thought to be positive for *TP53* mutation

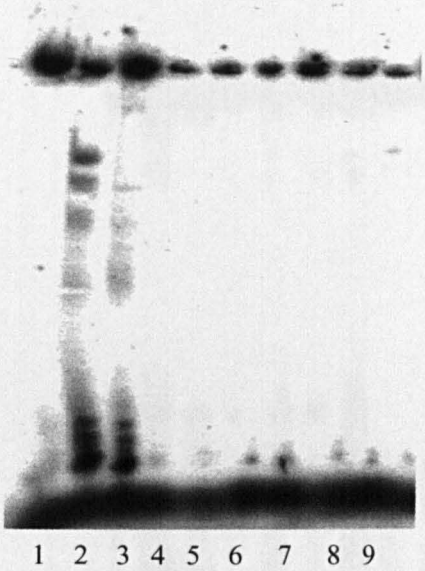


Figure A.6
TP53 experiment 1, showing positive results in lanes 2 and 3. The result in lane 7 was thought to be positive on initial imaging, but on closer examination this appears to be an artefact.
Lane 1 negative control
Lane 2 SW480
Lane 3 HT29
Lanes 4-6 tumours 1-3
Lane 7 tumour 5
Lane 8 tumour 6
Lane 9 tumour 10

A.4.3 Additional gel pictures for tumours positive for *BRAF* mutations

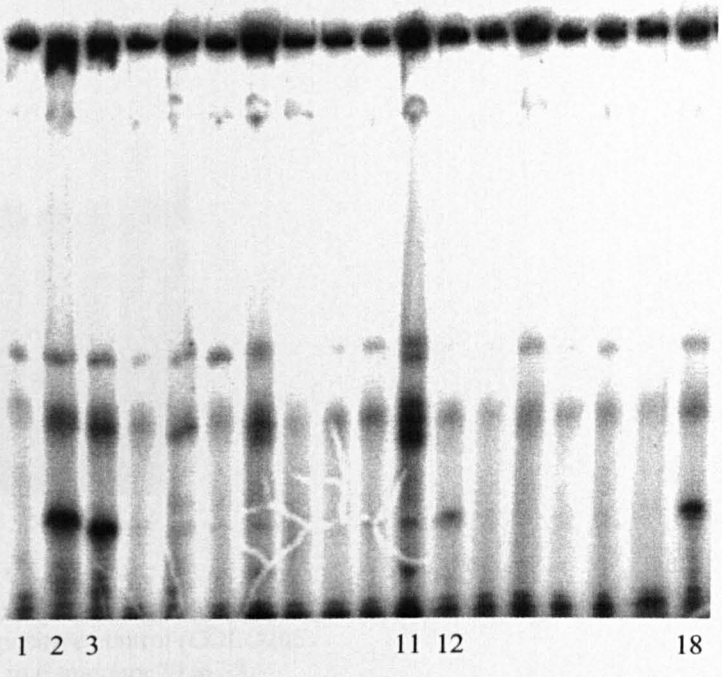


Figure A.7
BRAF experiment 9, showing positive results in lanes 2, 3, 12 and 18. The result in lane 11 is equivocal due to the presence of extra bands, and this tumour proved negative on subsequent testing.
Lane 1 negative control
Lanes 2 and 3 positive controls
Lane 4 tumour 8
Lanes 5 and 6 tumours 19 and 20
Lanes 7 to 13 tumours 22 to 28
Lane 14 tumour 42
Lanes 15 to 18 tumours 45 to 48

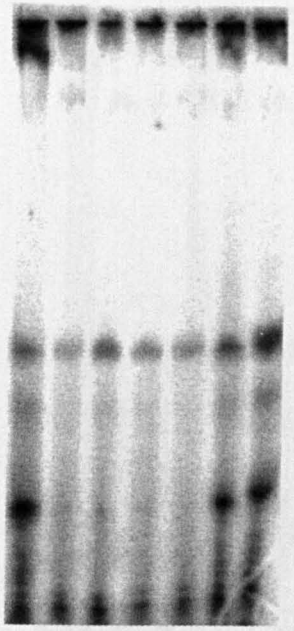


Figure A.8
BRAF experiment 13, showing positive results in lanes 1, 6 and 7.
Lane 1 positive control (COLO205)
Lanes 2 to 6 tumours 29 to 33
Lane 7 positive control (SW1417)

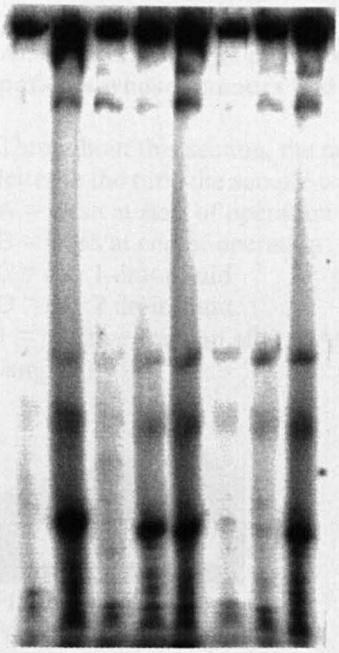


Figure A.9
BRAF experiment 14, showing positive results in lanes 2, 4, 5, and 8.
Lanes 1 negative control
Lanes 2 and 5 positive controls
Lanes 3 and 4 tumours 34 and 35
Lane 6 tumour 43
Lane 7 tumour 7
Lane 8 tumour 21

A.5 Gel Pictures for Positive Peritoneal Samples

A.5.1 Additional gel pictures showing positive results from peritoneal samples from patients whose tumours had *K-ras* mutations

Throughout this section, the number of a sample refers to the tumour number and the letter to the time the sample was taken.
A = wash at start of operation
B = wash at end of operation
C = day 1 drain fluid
D = day 2 drain fluid
+ = positive fraction after MACS separation (samples without this are non-separated samples)

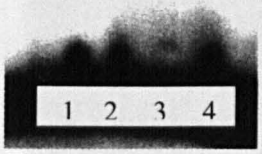


Figure A.10
Part of gel from experiment 3
Lane 1 positive control
Lane 2 tumour 13
Lane 3 13A+
Lane 4 13B+

Figure A.11 Experiment 22, showing positive results in lanes 1, 3, 6, 7 & 8
Lane 1 positive control
Lane 2 negative control
Lane 3 tumour 26
Lane 4 26B+
Lane 5 26C+
Lane 6 tumour 23
Lane 7 23A+
Lane 8 23B+

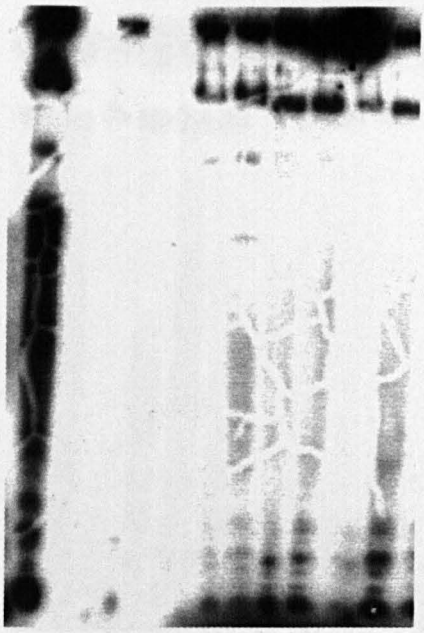


Figure A.12 Experiment 26. Clear positive results are seen in lanes 1, 7, 8 & 10
Lane 1 positive control
Lanes 2 & 4 empty
Lane 3 negative control
Lanes 5 & 6 36/37A (note that tumours 36 and 37 are from the same patient)
Lanes 7 & 8 36/37A+
Lane 9 36/37B
Lane 10 36/37B+

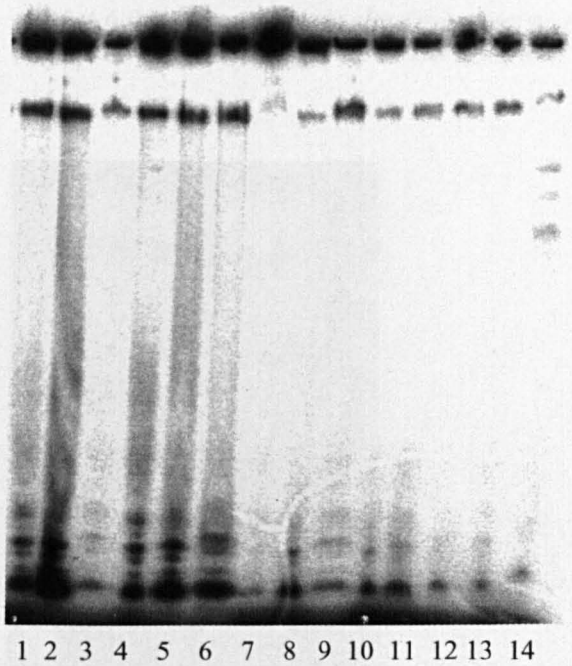
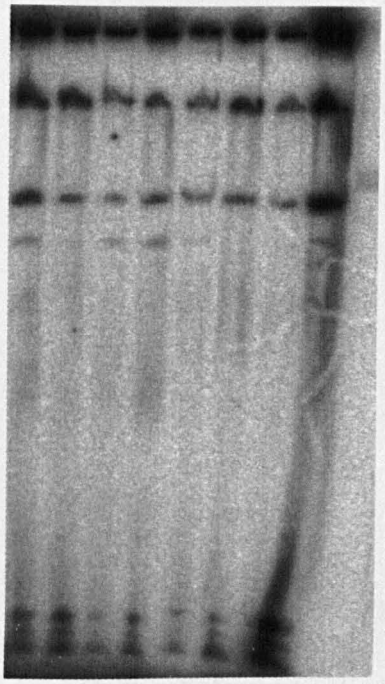


Figure A.13 Part of gel from experiment 27. Positive results in lanes 1, 2, 4- 6, and 8-11
Lane 1 32A
Lane 2 32A+
Lane 3 32B
Lanes 4 & 5 32B+
Lane 6 32C+
Lane 7 32D
Lanes 8 & 9 32D+
Lane 10 42A+
Lane 11 42B+
Lanes 12 & 13 42C
Lane 14 42C+

A.5.2 Additional gel picture showing positive results from peritoneal samples from patients whose tumours had *TP53* mutations



1 2 3 4 5 6 7 8

Figure A.14 Part of *TP53* experiment 16
Lanes 1 and 2 tumour 28
Lanes 3 and 4 28A+
Lanes 5 and 6 28B+
Lanes 7 and 8 28C+

A.5.3 Additional gel pictures showing positive results from peritoneal samples from patients whose tumours had *BRAF* mutations

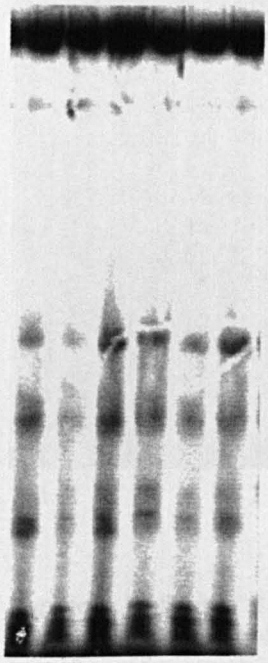


Figure A.15 Part of *BRAF* experiment 18
Lane 1 Spiked sample, 1 mutant cell in 1000 normal cells
Lane 2 Spiked sample, 1 mutant cell in 3200 normal cells
Lane 3 21A+
Lane 4 21B+
Lane 5 21C+
Lane 6 21D+
Clearly positive results are seen in lanes 1 and 3

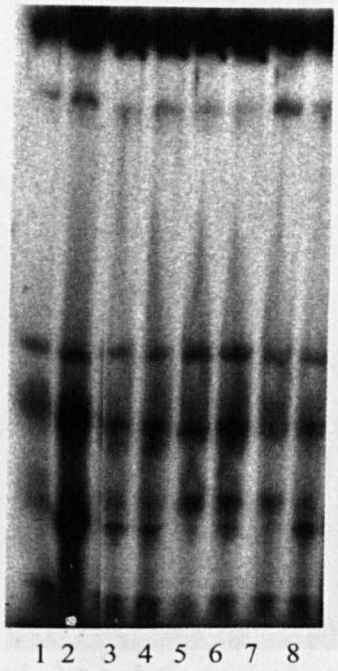


Figure A.16 Part of *BRAF* experiment 19
Lane 1 negative control
Lane 2 positive control
Lane 3 35A
Lane 4 35A+
Lane 5 35B
Lane 6 35B+
Lane 7 35C
Lane 8 35C+
Positive results are seen in lanes 2, 3, 4, 6 and 8.

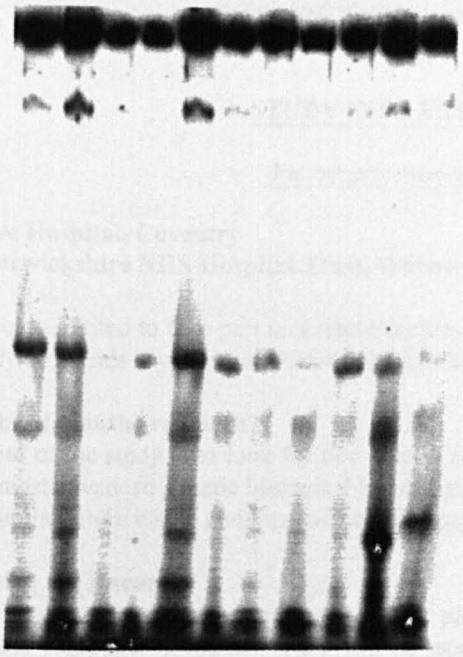


Figure A.17 Part of *BRAF* experiment 15
Lane 1 27A+
Lane 2 27B+
Lane 3 27C+
Lane 4 48A
Lane 5 48A+
Lane 6 48B
Lane 7 48B+
Lane 8 48C
Lane 9 48C+
Lane 10 spiked sample, 1 mutant cell in 100 normal cells
Lane 11 spiked sample, 1 mutant cell in 320 normal cells

SOUTH WARWICKSHIRE
GENERAL HOSPITALS NHS TRUST

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STUDY INTO FREE TUMOUR CELLS

Patient information sheet (April 2001)

Walsgrave Hospital, Coventry
South Warwickshire NHS Hospital Trust, Warwick

You are being invited to take part in a research study. Please take time to read this leaflet and to discuss it with family or friends if you wish. Please ask us if there is anything that you do not understand.

What is the aim of the research?
The purpose of the study is to look for free tumour cells close to the main tumour before and after surgery using the most advanced genetic biological techniques now available. The results of these studies may help us decide in the future which patients will benefit from further treatment in addition to surgery.

Who is doing the research?
A group of eleven consultant surgeons, several pathologists and a research worker in the Coventry, Warwick and Nuneaton hospitals. They will be working in conjunction with scientists in the Biological Sciences Department at Warwick University.

Which patients are in the study?
Patients having surgery on the oesophagus (gullet), stomach, pancreas and rectum (lower bowel). Most of these patients will be suffering from cancer but some patients will have other diseases.

Do I have to take part?
No. It is entirely your decision. If you do decide to take part you are free to change your mind at any time.

What does the study mean for me if I consent?
1 We will wash 100 ml of saline (salt water) around the operation site at the start and end of the operation and collect this fluid for analysis.
2 We will collect the fluid in your "drain bottles" on the first and second day after your operation and analyse it. This fluid is usually thrown away.
3 We will take a minute piece of the primary tumour from the specimen after surgery is completed . The major part of the tumour is analysed in the usual way.
4 We will monitor your progress over several years from your notes. This information will be kept confidential.
5 The fluid of some patients who have benign conditions will be used to check the accuracy of our tests.
6 **None of this is harmful or detrimental to your care.**
7 The results of our tests will be recorded in your notes. The results will not be used to change your care in any way because we do not know yet whether our tests are reliable. This is what we are trying to find out.
8 We will inform your own GP of the results if you wish.
9 This study has been accepted by the Warwick Area Health Authority Ethics Committee.

Your treatment will not be affected in any way if you do not wish to take part in the study. Thank you for reading this information sheet. If there is anything you do not understand please speak to the researcher or your own consultant surgeon.

The researcher (Karen Busby) can be contacted by pager. Phone 07654 369154.
If you decide that you would like to take part in this study we will ask you to sign a consent form. We will give you a copy of the consent form and a copy of this sheet to keep.

SOUTH WARWICKSHIRE
GENERAL HOSPITALS NHS TRUST

Department of General Surgery
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Study into free tumour cells
Patient consent form

1 I confirm that I have read and understood the information sheet for the above study and have had the chance to ask questions.

2 I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected

3 I understand that my medical notes may be looked at by responsible researchers involved with the study. I give permission for these people to have access to my records.

I agree to take part in the research study

Signature .....

Name .....

Date .....

I agree to let my GP know the results

Signature .....

I have explained the study to the above patient

Signature of researcher or consultant .....

Date .....

Research Doctor

Miss Karen Busby
Surgical Unit Office
Warwickshire NHS Trust
Lakin Road
Warwick CV34 5BW

Tel: 01926 495321 Ext 4155 or pager 07654 369151

Copy for patient Copy for researcher Copy for hospital notes

West Midlands South Strategic Health Authority
(Including Coventry, Warwickshire, Herefordshire & Worcestershire)

WARWICKSHIRE RESEARCH ETHICS COMMITTEE

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College Street,
Nuneaton
CV10 7DJ
Direct Line 02476 865244
Fax 02476 865058
E-Mail pat.horwell@geh.nhs.uk

17th September 2003

Dr. P. Murphy,
Consultant Surgeon,
South Warwickshire General Hospitals NHS Trust,
Warwick Hospital,
Lakin Road,
Warwick
CV34 5BW

Dear Dr. Murphy,

Warwickshire Local Research Ethics Committee
Approval Letter
RE 464 The evaluation of molecular biological techniques in the identification of tumour cells in the post operative drainage fluid after resection of oesophageal gastric and rectal caners

I am pleased to advise you that your application to participate as a Local Investigator in the above RE-approved study was reviewed by the Warwickshire Research Ethics Committee at their meeting on 31st January 2001 has now been approved. The Committee works in accordance with ICH/GCP guidelines

Please quote our RE reference number and title in all correspondence.

Yours sincerely

Pat Horwell ..... 17/9/03 .....

Paul Hamilton (Chairman) Date

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